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EDITED BY

SIMON FLEXNER, M.D. PEYTON ROUS, M.D.

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ON BIMOLECULAR LAYERS OF LIPOIDS ON THE CHROMOCYTES OF THE BLOOD.

By E. GORTER, M.D., and F. GRENDEL.

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(Received for publication, December 15, 1924.)

We propose to demonstrate in this paper that the chromocytes of different animals are covered by a layer of lipoids just two molecules thick. If chromocytes are taken from an artery or vein, and are separated from the plasma by several washings with saline solution, and after that extracted with pure acetone in large amounts, one obtains a quantity of lipoids that is exactly sufficient to cover the total surface of the chromocytes in a layer that is two molecules thick. Subsequent extractions with ether or benzene yield only small traces of lipoid substances.

We therefore suppose that every chromocyte is surrounded by a layer of lipoids, of which the polar groups are directed to the inside and to the outside, in much the same way as Bragg (1) supposes the molecules to be orientated in a "crystal" of a fatty acid, and as the molecules of a soap bubble are according to Perrin (2). On the boundary of two phases, one being the watery solution of hemoglobin, and the other the plasma, such an orientation seems *a priori* to be the most probable one. Any other explanation that does not take account of this constant relation between the surface of the chromocytes and the content of lipoids seems very difficult to sustain.

Technique.

1. All the glassware (centrifuge tubes, pipettes, funnels, filters, beakers, extraction apparatus) were made fat-free by concentrated sulfuric acid to which potassium dichromate had been added.

2. The reagents (water, benzene, acetone, ether, etc.) were twice distilled in an all glass distillation apparatus. The salt was ignited before use in a quartz crucible.

3. The blood was taken directly from an artery or a vein. The vessel was laid free and a needle twice boiled in doubly distilled water to which first 1 per cent

soda, and then 0.5 per cent potassium oxalate had been added, was introduced into it. The first stream of blood was discarded to avoid the possibility of error from contamination with the fat of the subcutaneous tissue. The next portion was then permitted to flow into a small stoppered weighing bottle, containing 0.5 per cent potassium oxalate. In the case of the goat and the sheep the jugular vein was directly punctured through the skin but in this case the stream of blood was permitted to flow for some time, so as to wash the needle clean of all contaminating fatty substances before a measured quantity was received in our glass vessel. In human subjects the same procedure of puncturing the vein through the skin was followed.

4. After mixing, 10 cc. (or in later experiments 1 cc.) of blood were pipetted into a centrifuge tube of 60 cc. and three or four times washed with 50 cc. salt solution (0.9 per cent) in the usual way.

5. The extraction was performed with acetone during 48 or 72 hours. Large quantities were used.

After several extractions, the acetone was filtered into a glass beaker and the liquid evaporated on a water bath. This procedure was the most difficult part of the operation because loss was very liable to occur at this time. The residue was finally taken up in benzene and filtered into a measuring flask of 50 cc., when 10 cc. of the blood had been used, or in a tube marked at 2.5 or 5 cc., when 0.5 or 1 cc. had been taken. Just before each determination the liquid was made up to the mark with benzene.

Determination of the Surface Occupied by the Lipoids Spread Out in a Monomolecular Layer on Water.

Langmuir (3) has demonstrated that fats and fatty acids spread in a monomolecular layer when they have been dissolved in benzene and a few drops of the solution are placed on a large surface of water. Adam (4) has slightly modified the apparatus originally described by Langmuir. We have made use of Adam's modification. The benzene solution was delivered out of a calibrated 0.1 cc. pipette.

Now, it has been shown that the molecules of a fatty substance spreading on a water surface do not exert any pressure in a direction parallel to the surface before the condition is arrived at that they form precisely a monomolecular film, in which latter they come to be arranged in a vertical position. In the Langmuir-Adam apparatus the water surface chosen is so large that sufficient room is provided to the molecules so that they are not in close contact with each other. By the displacement of a strip of copper on which a balance is mounted one is able to determine the precise moment at which the molecules

begin to exert a pressure in a horizontal plane, and by placing different weights on the pan of the balance, it is possible to compensate and to measure this pressure. The reduction of the size of the surface is obtained by moving a glass strip covered with a thin layer of paraffin oil over the edges of the copper tray, which are covered as well with paraffin oil. As soon as the molecules are in close contact in a layer exactly one molecule thick, the balance moves out of the equilibrium position. By placing small weights on the balance one is able to compress the layer without much further reduction of the size of the surface, till suddenly by increasing the weight the layer is disturbed and equilibrium of the balance is no longer obtained. The dimensions of the surface are measured with a ruler.

We always began with the determination of the surface contamination. By placing 50 mg. in the pan of the balance and moving the glass strip from a distance of about 30 cm. we were able to determine that it hardly ever exceeded 0.5 cm. at room temperature.

From a pipette 0.1 cc. of the benzene solution of the lipoids of the chromocytes was blown onto the surface of the water in the tray and by moving the glass strip the point was noted at which the balance began to move, 50 mg. being the weight in the pan. The pressure exerted on each cm. of the layer was 2 dynes per 50 mg. weight in the pan.

Determination of the Number and the Dimensions of the Chromocytes.

The number of chromocytes was determined by filling the *mêlangeur* as soon as possible from the weighing bottle containing the blood, and by counting in the counting chamber of Bürker the cells in 80 small squares, each measuring 1/4,000 c.mm. The surface of the chromocytes was evaluated from blood smears on slides, coloured by Pappenheim's panoptical dye. With the aid of a drawing prism of Zeiss 40 to 50 chromocytes were drawn on millimeter paper. By taking account of the magnifying power of the microscope one was able to measure the dimensions of the cells in a horizontal and a vertical direction.

The surface of the cells was derived from these numbers by making use of Knoll's (5) formula that in chromocytes having the form of a disc (a form that is taken by all chromocytes that are spread on glass) the surface is $2D^2$(D being the diameter).

The total surface of the chromocytes from 1 to 10 cc. blood was easily obtained by multiplying the number of cells by their surface.

SUMMARY OF RESULTS.

We have examined the blood of man and of the rabbit, dog, guinea pig, sheep, and goat. There exists a great difference in the size of the red blood cells of these animals, but the total surfaces of the chro-

TABLE I.

	Animal.	Amount of blood used for the analysis.	No. of chromocytes per c.mm.	Surface of one chromocyte.	Total surface of the chromocytes (*a*).	Surface occupied by all the lipoids of the chromocytes (*b*).	Factor *a*:*b*.
		gm.		*sq. μ*	*sq. m.*	*sq.m.*	
1	Dog A	40	8,000,000	98	31.3	62	2
2		10	6,890,000	90	6.2	12.2	2
3	Sheep 1	10	9,900,000	29.8	2.95	6.2	2.1
4		9	9,900,000	29.8	2.65	5.8	2.2
5	Rabbit A	10	5,900,000	92.5	5.46	9.9	1.8
6		10	5,900,000	92.5	5.46	8.8	1.6
7		0.5	5,900,000	92.5	0.27	0.54	2
8	" B	1	6,600,000	74.4	0.49	0.96	2
9		10	6,600,000	74.4	4.9	9.8	2
10		10	6,600,000	74.4	4.9	9.8	2
11	Guinea Pig A	1	5,850,000	89.8	0.52	1.02	2
12		1	5,850,000	89.8	0.52	0.97	1.9
13	Goat 1	1	16,500,000	20.1	0.33	0.66	2
14		1	16,500,000	20.1	0.33	0.69	2.1
15		10	19,300,000	17.8	3.34	6.1	1.8
16		10	19,300,000	17.8	3.34	6.8	2
17		1	19,300,000	17.8	0.33	0.63	1.9
18	Man.	1	4,740,000	99.4	0.47	0.92	2
19		1	4,740,000	99.4	0.47	0.89	1.9

mocytes from 0.1 cc. blood do not show a similarly great divergence, because animals having very small cells (goat and sheep) have much greater quantities of these cells in their blood than animals with blood cells of larger dimensions (dog and rabbit).

We give all the results of our experiments, omitting only those in which we were unable to avoid losses in the procedure of evaporation of the acetone.

It is clear that all our results fit in well with the supposition that the chromocytes are covered by a layer of fatty substances that is two molecules thick.

BIBLIOGRAPHY.

1. Bragg, W. H., and Bragg, W. L., X rays and crystal structure, New York, 4th edition, revised and enlarged, 1924.
2. Perrin, J., *Ann. phys.*, 1918, x, series 9, 160.
3. Langmuir, I., *J. Am. Chem. Soc.*, 1917, xxxix, 1848.
4. Adam, N. K., *Proc. Roy. Soc. London, Series A*, 1921, xcix, 336; 1922, ci, 452, 516.
5. Knoll, W., *Arch. ges. Physiol.*, 1923, cxcviii, 367.

THE INFLUENCE OF A LOW ELECTROMOTIVE FORCE ON THE ELECTROPHORESIS OF LYMPHOCYTES OF DIFFERENT AGES.

By HAROLD A. ABRAMSON, M.D.

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(Received for publication, December 26, 1924.)

McCutcheon has reported (1) that small lymphocytes kept at 37°C. exhibit spontaneous movement which increases with the time that the cells are kept outside the body. McCutcheon allowed a drop of blood from the finger tip to spread between a glass slide and cover-slip. Then, surrounding the rim of the cover-slip with vaseline, he was able to study his preparation over a period of hours. The figures given in McCutcheon's paper show that with this technique the average spontaneous velocity of small lymphocytes increases from 4 micra per minute during the 1st hour, to over 10 micra per minute, reaching 15 micra per minute, after they were outside the body over 5 hours. McCutcheon does not state what direction of movement the cells took. Nor does he mention the changes in the blood film as a result of clotting or fibrin precipitation as a possible influence on lymphocytic movement. It would be interesting to note the velocity of movement during a period when the changes due to clotting are presumably at a minimum. This probably would be during the first 10 minutes of study, omitting, of course, the first movements during settling of the cells.

In 1922 a series of experiments were done to determine the influence of a low electromotive force on the movements of white blood cells, particularly small lymphocytes. The type of cell studied was similar to that studied by McCutcheon. A low electromotive force was used in order to compare with this electromotive force the electromotive forces passing between injured and relatively normal tissues. If these two could be compared, it could then be inferred that perhaps one of the factors which brings white blood cells to a point of injury may be this "current of injury." This has been discussed somewhat in detail elsewhere (2).

The cell in which the movements of the white blood cells were studied was constructed as follows:

Unglazed porcelain non-polarizable boot electrodes were set in a heavy glass slide, toe to toe, and then ground down so that the levels of the toes and glass were about the same. After placing a small drop of the uppermost layer of cells of centrifuged defibrinated blood on the glass block between the electrodes, a round cover-slip of 1 cm. diameter was placed over the drop which then spread out, just reaching the rim and simultaneously connecting the two electrodes. The edge of the cover-slip was instantly surrounded by a low melting liquid paraffin which solidified almost immediately. There was then ready to be studied on a specially constructed sliding warm stage at body temperature, a very thin film of blood cells in serum.

It is important to discuss some of the errors introduced by the use of an electrophoresis chamber of the construction described. In brief, the "true mobility" of the particles in suspension when under the influence of an electromotive force is the average mobility at all levels in the cell because of an independent flow of positively charged water near the glass surfaces (3). Since the cell is a closed system, there will necessarily be a slight anodal flow in the mid-regions of the cell. Thus, a negatively charged particle will be accelerated in the mid-regions of the cell and retarded near the surfaces of the glass.

A second flow of water within the system is caused by the charge on the water incidental to the presence of the porous porcelain electrodes. These electrodes permit the passage of water through the capillary spaces of the electrodes. Whatever the electrode's influence is due to, whether to the presence of a porcelain membrane, or, in addition, to a porcelain-protein membrane at the junction of the electrode with the serum, the water would be positively charged in relation to the membrane. That is, under the influence of a potential gradient there would be a cathodal flow of water through the system and across the field of observation, thus retarding the migration of a particle migrating toward the anode. For a more complete discussion of this subject the work of Briggs (4) and the numerous papers of Loeb in *The Journal of General Physiology* may be consulted.

In the experiments to be described, the lymphocytes studied were only those which moved in a nearly straight line across the field. The direction of migration was only toward the anode. *No lymphocytes at any level were ever observed to go toward the cathode.* The cells

studied were those in the mid-regions of the chamber, those resting on the glass hardly showing any "motility." Hence the figures in Table I are not the "true mobility" of the lymphocytes for the P.D. of 0.8 volt per cm. which was used, but they are approximations of velocity which may *not* be substituted in the Helmholtz-Lamb equation to determine the P.D. between lymphocytes and serum. The figures in Table I may be compared because of the large distances travelled, the constancy of technique, and the thinness of the film which made up the electrophoresis chamber.

TABLE I.

The Influence of an Electromotive Force of 0.8 Volt per Cm. (Current 1/25 Milliampere) on the Speed of Anodal Migration for Different Ages of Small Lymphocytes in Serum; Kept in Ice Box until Studied unless Used Immediately.

Age of lymphocytes.	Distance travelled.	Time.	Speed per min.
	micra	*min.*	*micra*
Used immediately.	185	15	12+
" "	180	14	12+
2 hrs.	210	15	14
2 "	260	15	17+
3 "	285	30	9+
6 "	410	34	12+
6 "	410	40	10+
6 "	410	31	13+
6 "	105	8	13+
30 "	205	12	17+
30 "	410	25	16+

The blood from which the lymphocytes studied were obtained, was drawn from the arm vein of a presumably healthy adult male, and after defibrination and centrifugation was kept in the ice box until used, unless the cells were studied immediately. In these same series of experiments no constant direction of migration was noted for polymorphonuclear leucocytes. However, Eliasoph (5) using platinum electrodes has noted that polymorphonuclear leucocytes, when studied in splenic tissue and gelatin suspensions, migrate toward the anode. From this work it would seem that polymorphonuclear cells are also negatively charged. Since this author used a compara-

tively high electromotive force, the magnitude of which is not mentioned in P.D. per cm., and since there was evidence of chemical change about the electrodes, these results cannot properly be considered in the theoretical considerations to follow.

With the exception of the 3 hour velocity and one of the 6 hour velocities, the speed of migration of the lymphocytes varied from 12+ to 17+ micra per minute. This variation is to be expected because of the experimental conditions. Therefore it may be considered that the length of time the lymphocytes were kept outside the body in serum in the ice box did not appreciably influence the speed of the anodal migration. It is quite interesting to note that the straight line velocity of small lymphocytes under the conditions studied is about the same as that reported by McCutcheon for "spontaneous velocity." It should be remembered that the increase in "spontaneous velocity" with time from 4 micra to 15 micra per minute was noted in McCutcheon's study of a whole blood film. The possible influence of clotting has been considered.

While the electromotive force was passing, lymphocytes in motion had practically always straight line motility (Fig. 1) and with interruption of the current, immediately ceased their movements. Or with reversal of the current they came to a dead stop, and immediately started to migrate to the new anode.

The question arises whether in the living animal tissues the electromotive forces which pass between injured and relatively normal tissues are of the same order of magnitude as the experimental electromotive force of 0.8 volt per cm. When the tissues are injured, the many physicochemical changes at the site of injury produce a system which makes the injured area electropositive (6). Thus the injured area may be considered similar to the anode and the direction of migration of the lymphocytes be correlated with the suggestion that the electromotive force is partially responsible for the cellular part of the inflammatory reaction as far as direction is concerned. Measurements of the "current of injury," of course, have not measured the P.D. between cells which were normal and injured, but have determined rather the P.D. between fascial plane and injured muscle or nerve (6). Hence, although electromotive forces of 0.08 volt have been reported, the actual P.D. per cm. between the injured and

uninjured tissue is not known. Since some cut surfaces have been found to be (by the authors cited above (6)) at about the same electrical potential as the uninjured fascial surface at the moment after cutting, it may be inferred that the difference in potential between injured and uninjured tissues may be of a somewhat similar order of magnitude as that measured between fascial surface and injured surface. The many changes in physicochemical make-up of injured

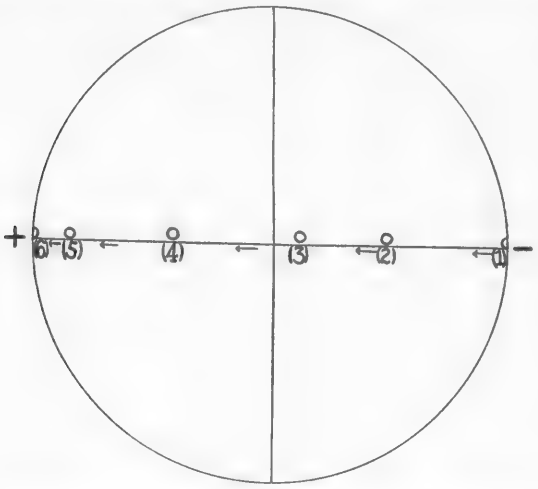

FIG. 1. Schema to show course of lymphocyte and method of determination of speed. Diameter of field 410 micra. Cross lines on eyepiece. Lymphocyte entered field at (1), time 4:01, was at (3) at 4:11, and crossed out of range at 4:35 (6). The straight line movement was occasionally slightly above or below the line in other determinations but in general was similar to the migration depicted in the figure. The time taken to migrate 410 micra was 34 minutes. In this experiment therefore, the mean rate was 12+ micra per minute.

tissues most probably produce a difference of potential between limiting border of the zone of injury and the *nearest* relatively normal cells of at least 10 millivolts. If the nearest relatively normal cells are 1 mm. away, the P.D. per cm. would be 100 millivolts or 0.1 volt per cm. This would place the electromotive force used in these experiments in the same order of magnitude as those existing in living tissues.

SUMMARY AND CONCLUSIONS.

1. Small lymphocytes in serum at 37°C. migrate toward the anode under experimental conditions described.

2. The electromotive force of 0.8 volt per cm. under the same conditions produced a straight line velocity which approximated that reported by McCutcheon for spontaneous velocity after his preparation had been studied over 5 hours.

3. Keeping the lymphocytes on ice up to 30 hours did not produce an appreciable difference in velocity of straight line migration from that speed determined if the cells were studied immediately.

4. The possible relationships between the type and order of magnitude of the electromotive force passing between injured and normal tissues and the electromotive force used experimentally are briefly discussed.

The author desires to express his sincere thanks to Professor H. W. Farwell of the Department of Physics, Professor H. B. Williams of the Department of Physiology, and Professors W. C. Clarke and A. P. Stout of the Department of Surgery, for the many courtesies given him during the course of this work.

BIBLIOGRAPHY.

1. McCutcheon, M., *Am. J. Physiol.*, 1924, lxix, 279.
2. Abramson, H. A., *Am. J. Med. Sc.*, 1924, clxvii, 702.
3. Northrop, J. H., *J. Gen. Physiol.*, 1921–22, iv, 629, (who also gives other references).
4. Briggs, T. R., *J. Phys. Chem.*, 1917, xxi, 198.
5. Eliasoph, B., *Z. ges. exp. Med.*, 1924, xli, 317.
6. Du Bois-Reymond, E., Untersuchen über thierische Elektricität, Berlin, 1848. Hermann, L., Handbuch der Physiologie der Bewegungsapparate, 1879, i, 192. Biedermann, W., *Ergebn. Physiol.*, 1903, ii, pt. 2, 264.

THE RELATIVE REACTION WITHIN LIVING MAMMALIAN TISSUES.

III. Indicated Differences in the Reaction of the Blood and Tissues on Vital Staining with Phthaleins.

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(Received for publication, January 29, 1925.)

In the present paper and others to follow some color phenomena noted in animals stained during life with phthalein indicators will be described. It is a curious fact that these indicators have not been utilized heretofore for a general vital staining, in view of their multifarious applications to the study of body fluids. The findings in the stained animals will be presented objectively for the most part. Interpretation of them can be but tentative at best, in the present state of knowledge.

To come at the reaction within living cells and organs by means of indicators, one must have available substances of a sort which will penetrate readily, and not interfere with the normal activities; which will stain so intensely as to prevail on the color of the tissues themselves, yet not give false information. These are conditions difficult to satisfy. And with each indicator it will be necessary to determine exactly what is stained. No more need be said to show that the task is one of great complexity.

In previous communications the results of vital staining with litmus have been described.[1,2] The coloring matter diffuses poorly, and becomes largely segregated in red form within intracellular granules. The acidity of these granules, as indicated with litmus and some of the phthaleins of a more acid range, is far from negligible.[2] There are certain tissues which appear to stain diffusely with litmus, and these are mostly rendered blue by it. But in the case

[1] Rous, P., *J. Exp. Med.*, 1925, xli, 379.

[2] Rous, P., *J. Exp. Med.*, 1925, xli, 399.

of one at least, the liver, signs have been found that some portion of the indicator unsegregated within granules is pink.

The phthaleins are highly diffusible, as will be shown, and maintain their characteristics as indicators when introduced into living animals. Most of them are well tolerated by mice in amounts which color these creatures brightly. The staining is far more general than that with litmus and ordinarily is diffuse, though localization in the cell granules can, on occasion, be brought about.[2] Elimination, which occurs into the bile and urine, is, like the spread of the dyes through the body, extremely swift, and the coloration of the tissues usually is lost within a few hours.

Method.

Half-grown to adult mice were employed. The sodium salts of the indicators (Hynson, Westcott and Dunning) were prepared according to Clark's directions,[3] immediately prior to injection, and dissolved in 0.9 per cent saline solution. No sterilization was attempted. Ordinarily, each 10 cc. of the fluid contained 0.1 gm. of the original indicator, or 0.2 gm. in the case of such as were highly soluble. When thus made up, all of the solutions except those of brom phenol blue and brom cresol green had colors showing that the indicators were still in the acid form. A very little N/20 NaOH was added, just sufficient to change the hue to the alkaline side and, when the range permitted, to the color indicative of pH 7.4. It was found that subcutaneous injections of the material yielded but a weak staining because elimination nearly kept pace with absorption. When, however, the dyes were thrown into the peritoneal cavity, in amounts ranging from 0.5 to 3.0 cc., there resulted a pronounced coloration of the whole animal. As a rule the examination was carried out when this had reached a maximum, the mouse being put under ether and the tissues inspected. In order to avoid, so far as possible, the complication of acidosis incident to prolonged anesthesia, the dissections were made rapidly, and were in many instances confined to a few organs. The skin incision was carried around the site of injection to avoid a possible contamination with free indicator. The instruments were cleansed in boiling water and dried upon gauze that had been washed free of soluble material.

Preliminary observations brought out the fact that changes in the reaction of some of the tissues take place practically at once on exposure of them to air. For this reason, as a rule, the dissections have been carried out with the body immersed in washed paraffin oil in a tray with a layer of solid paraffin at the bottom. The oil had been tested and shown not to affect the indicators even in unbuffered solutions. The colors glow with a special brightness in it. The tissue bits for microscopic examination were clipped or sliced away and placed in oil between a slide and cover-glass of mica. Pieces of the larger organs were

[3] Clark, W. M., The determination of hydrogen ions, Baltimore, 2nd edition, 1922.

sliced in the tray with a Valentine knife. The inspection of color was carried out as swiftly as possible, first with the naked eye, then under low and high magnifications. Daylight was used throughout. In most instances there was a little of the indicator fluid still free on the peritoneal surfaces at the time of the examination, and, when necessary, this was taken off with gauze. It never interfered with the appraisal of the hue in the interior of the viscera.

The observations of the present paper and that to succeed it will be reported in the order in which the phthaleins stand in series, from thymol blue in its alkaline phase to brom phenol blue which has a range well to the acid side. The specimens of indicator employed have been checked up in Sörensen's double phosphate buffers, kindly provided and controlled electrometrically by Dr. D. R. Drury; and, with the exceptions of brom phenol red and chlor phenol red, they have been found to possess the ranges generally accredited to them. Checking has special importance in the case of brom cresol purple which may be so altered when the alkaline salt is improperly made that its range is narrowed and shifted toward the alkaline side.

The available range of all of the phthaleins is greatly narrowed when they are diffused throughout the body, for the reason that only outspoken color differences, not varying intensities of the same hue, can then be depended upon. For, needless to state, observed local differences in intensity may be the result of differing local concentrations of the dye, not of differences in reaction. The colors also are influenced by this factor, dilute solutions of an indicator at a reaction close to the middle of its range having often a different hue from more concentrated ones. The difficulty thus introduced was circumvented by comparing the hue of each stained tissue with those of the tubes of a series of buffer solutions containing the indicator in approximately the color intensity manifested by the tissue. For the purpose several graded sets of buffer mixtures containing different amounts of each indicator were requisite. Often a comparison was possible with the color chart given by Clark.[3] It may be recalled that for each hue depicted in this last the pH is given to which said hue corresponds under controlled conditions. The use I shall make of these pH numbers in recording the color of stained tissues must not be taken to imply a conclusion that the latter had the precise reactions for which these numbers ordinarily stand. They will be employed merely as index symbols, save where otherwise stated. To minimize the error introduced by the native hue of the tissues a staining sufficient to prevail over this was induced. Yet, needless to remark, it had always to be taken into account.

Control Observations.

In view of the great diffusibility of the phthaleins and the intense coloration they produced in the animals there could be no doubt that the dyes passed from the blood stream into the tissues generally.

Yet direct tests of the point seemed advisable. Accordingly, mice were injected with the amounts of cresol red, phenol red, and brom cresol purple employed in the experiments proper, and when the staining had reached its maximum they were rapidly etherized, the inferior vena cava severed just above the diaphragm, and warm Ringer's solution was slowly injected into the left ventricle of the exposed and beating heart. As result the animal exsanguinated itself in the course of a minute or two and washed itself out with fluid propelled by the ventricular contractions. The bloodless ears, lungs, connective tissue, epidermis, liver, and kidney were found in every case to be notably stained with indicator. There was less in the voluntary muscles, and still less in the heart muscle, though even here some was demonstrable. The parenchyma of the testicle was looked at in a few cases and found stained; but no general examination of the organs was undertaken. The presence of the indicators was of course to be expected in the liver and kidneys which excrete them.

According to Clark and Lubs[4] the phthaleins manifest much resistance to chemical influences such as they would be likely to encounter within the body. That the hues which they assume when distributed through the tissues are an expression of their behavior as indicators is plain from the characteristic alterations to be noted when the reaction is in any way changed. But it is essential to know whether the color distinctions remain the same in the body as when the indicators are added as such to buffer solutions. This is readily demonstrable. Animals given an indicator are exsanguinated under ether, and various of the stained organs are thinly sliced and the slices squeezed between dry, neutral gauze to remove still more of the blood. It would have been better to have employed organs washed out with salt solution. The slices are placed in water for a few moments in order to remove as much as possible of what is left of the tissue fluids, and are then distributed in a graded series of buffer solutions. I used Sörensen's double phosphate mixtures. Both in the case of phenol red and brom cresol purple, the most valuable phthaleins for tissue work, the hues assumed by the indicator diffusing out into the buffers corresponded absolutely with those obtained on

[4] Clark, W. M., and Lubs, H. A., *J. Bact.*, 1917, ii, 1, 109, 191. Clark, W. M., personal communication.

adding the phthaleins directly to a similar series of solutions, as furthermore with those given in Clark's chart. Brom cresol purple excreted into the bile retains the typical range.

The Staining with Thymol Blue.

Thymol blue in its alkaline phase is yellow in dilute solution at pH 8.2, but with increasing degrees of alkalinity changes until at about pH 9.4 it has become sufficiently blue for the color to dominate presumably over that proper to most tissues, should they contain it. The indicator is one of the few that is poorly tolerated. Mice receiving enough of the alkaline salt to color them markedly succumb as a rule within the next 24 hours. Somewhat smaller amounts appear to interfere little with health. The tinting develops rather slowly, reaching a maximum only after several hours and may not have wholly disappeared by the next day. A deep yellow urine and yellow feces are voided, both of which turn blue with alkali. Nowhere in the body is the blue color found. When the staining is at its height, the hairless surfaces are pronouncedly yellow like the tissues disclosed by dissection. The question of the precise distribution of thymol blue within the body need not be dwelt upon since tests with the other phthaleins show clearly that none of the tissues of the mouse is alkaline enough to come within its range.

Cresol Red.

Cresol red, when dilute enough to be comparable with the color scale of Clark, is yellow at about pH 7.2, becoming gradually red on alkalinization, until at approximately pH 8.4 this color is outspoken. The red component becomes more evident with increasing concentration of the dye, and, when this is ten times that just considered, the hue in a buffered solution (Sörensen) at pH 7.55 is damson-red, at 6.98 ruddy orange, and only at 6.24 is a clear orange attained. The amount of cresol red in the tissues, when animals are properly stained, permits of a direct comparison with Clark's table, whereas in the blood so much more is present that to gauge its hue properly recourse must be had to graded buffer solutions containing the phthalein in considerable amount.

Mice receiving 1 cc. of 1 per cent cresol red become a ruddy yellow

within an hour while within another the staining has largely faded and by next day has wholly disappeared. The urine is sometimes deep yellow and again intensely red. The animals at no time appear disturbed. On close scrutiny of the ears it can be seen that the red element in the coloration is confined to the blood (and lymph ?). For when the ear is rendered bloodless by gentle pressure between glass slides, it appears clear yellow. The same holds true for the subcutaneous tissue adherent to a skin flap everted under light ether anesthesia with the animal submerged in oil. A like yellow coloration is to be found almost everywhere, save in the brain and nerves which are not stained. In special the connective tissue, adipose tissue, tendons, cartilages, epidermis—except for the horny layer—the lymph nodes, kidney, spleen (Malpighian bodies), liver, gall bladder wall, pancreas, heart muscle, and voluntary muscle are all yellow with the dye, as is the medulla of the suprarenal and the more vascular regions of the bones. Suprarenal cortex and laminated bone appear unstained. The yellow color, if encountered under controlled conditions in a buffered solution, would mean a reaction at or below pH 7.2. The finding does not stand alone. Tests with indicators of less alkaline range have given results in close agreement with it, as will be shown.

Phenol Red.

Mice behave normally when deeply colored with phenol red,—as after the injection of 1 cc. of a 2 per cent solution into a 30 gm. animal; and no impairment of health occurs subsequently. The staining is at its height within an hour and has almost disappeared within another. Ruddy to crimson urine is voided. The indicator is clear yellow in dilute solution at pH 6.6, changing toward old rose with gradual alkalinization until at pH 7.8 the hue is outspoken. In concentrated solutions the rosy element extends much further down the pH scale, as in the case of cresol red. It follows that the color differentiation of the tissues is not well seen when vital staining is intense.

In stained animals the hairless surfaces appear pink; yet on scrutiny the color is seen to be mixed with yellow, a state of affairs most evident

in the ears by transmitted light. Wherever vessels are few, as near the margin of the auricle, the color is yellow, and, where many, a deep pink. Pressure sufficient to drive out the blood leaves the tissue yellow. Clippings of the epidermis of the tail, feet, or abdomen are all brilliant yellow, save in the horny layer which is unstained. The adipose tissue attached to some specimens is also bright yellow. In lightly etherized animals with the body submerged in oil the only stained fabrics which do not appear pure yellow are those of the tendons and connective tissue. Everywhere throughout the body the latter is yellow-pink to ruddy orange, according to the amount of phthalein present, the hue corresponding ordinarily to that of pH 7.2 in Clark's scale, though often it is more yellow, a slightly pinkish yellow, as the tendons regularly are. The brain and nerves are not colored; and the distribution of the dye in the other organs closely accords with that of cresol red, so there is no need to designate those rendered yellow by it. Occasionally the cortex of the kidney is red, but ordinarily it is yellow, the individual differences being dependent, doubtless, on functional conditions.[5] The prevalence of yellow throughout the body is the more noteworthy because the blood, as viewed within the vessels, appears either old rose or damson-red, depending on how much indicator is present. The inguinal lymph glands, inspected *in situ*, have a rosy interior, but when they are rapidly excised and broken open between mica slides, by pressure the color is found to be confined to the lymph and blood, the tissue proper to the gland being a clear yellow.

The coloration with phenol red, if accepted at its face value, would mean that none of the stained organs except the connective tissue and tendons can have a pH greater than 6.6. The hue assumed by the blood on the other hand would indicate that it is relatively alkaline, a finding in agreement with what has been noted of cresol red animals. But the differing concentrations of the extra- and intravascular indicator may conceivably be responsible for much of the color difference. In some further, more precise, observations account was taken of this factor.

[5] Stieglitz, E. J., *Arch. Int. Med.*, 1924, xxxiii, 483.

Effects of Ether Anesthesia on the Vital Coloration.

The animals from which stained blood was to be taken were placed under ether. When light anesthesia had been rapidly induced, no change was to be noted in the color of the ears, tail, or other hairless surfaces; but when it was heavy, or had been maintained for some time during a detailed inspection of the organs, the body surfaces showed noteworthy alterations in hue indicative of a changed reaction of the blood. In cresol red animals the vascular portions of the ears no longer appeared ruddy yellow but were yellow, the vessels now showing merely as fine orange lines, not red ones as previously. An identical alteration was to be noted throughout the blood of the organs. This phenomenon, indicative of an acidosis, was not quite so plainly to be remarked in phenol red mice, presumably because of the circumstance that the range of this indicator lies further to the acid side, the alteration toward acidity during life being never sufficient to convert the color completely from damson or rose to yellow. The pink of the hairless surfaces was altered by the anesthesia to, at most, a pinky yellow. With the brom phenol red recently described by Cohen, my specimen of which had a range only slightly different from that of phenol red, being yellow at pH 6.47 when in dilute solution, and old rose at 7.38, the changes were much the same. The ears of the stained animal were ordinarily pink, with an orange admixture where there was little blood, but during prolonged ether anesthesia they became yellow save for a few of the larger vessels, which retained the red hue. With the phthaleins of a more acid range color changes fail to occur during anesthesia.

The development of acidosis during ether anesthesia is, of course, a well known phenomenon. However, its manifestations in the stained mice have an interest both as proving that the circulating phthaleins maintain their indicator characteristics, and as pointing to a source of error in animals examined while anesthetized. The acidosis is evident even when the breathing is so hastened and exaggerated as to give the impression that an overventilation must be taking place. The question as to the extravascular implications of the change in blood reaction cannot be answered with the red phthaleins now under consideration, owing to the fact that nearly all of the tissues have a reaction beyond their range on the acid side.

Connective tissue is an exception in that it is colored yellow-pink with phenol red. I have not been able to observe any certain alteration in its hue during prolonged anesthesia unaccompanied by asphyxia. In the experiments now to be detailed, to compare the blood and tissue reactions, the etherization has been light and brief, yet some slight change in the color of the hairless surfaces has been not infrequent.

Differing Coloration of the Blood and Tissues.

General Experimental Procedure.—Mice stained with cresol red or phenol red were lightly etherized, placed at once in the oil bath, and the skin rapidly stripped back from over abdomen and chest. The color of the subcutaneous tissues was noted *in situ;* the thorax was laid open with scissors; and blood was aspirated from the right ventricle directly into one, or, when possible, two tubes containing a column 1½ to 2 cm. long of paraffin oil. The tubes had a bore of about 0.35 cm. and were of "Perfection" glass, which contains practically no free alkali. An end had been drawn out into a wide-angled but sharp point to thrust into the heart. Of each pair of tubes one had been rinsed in twice distilled water, just before oil was drawn into it, and the other had been coated with paraffin and then rinsed prior thereto. The blood never came in contact with air. Immediately that it had been withdrawn the unsealed sharp tips of the pipettes were thrust deep into rubber stoppers and they were centrifuged in chilled containers for 2 or 3 minutes at high speed. In this way a clear plasma was obtained which was compared, at room temperature, in buffered solutions of indicator. In the case of cresol red several series of such solutions were used containing different concentrations of the indicator; and a match in color and intensity for the blood plasma was found by taking up the appropriate buffer solution into a tube like that containing this latter. Duplicate plasma specimens were found to have always the same hue. The presence of dissolved hemoglobin as a complicating factor was ruled out with the spectroscope, and the influence of oil, paraffin, and glass by preliminary tests with dilute watery indicator solutions introduced into the tubes. Even after 24 hours at room temperature the stained plasma, now clotted of course, had changed but faintly in hue, toward the acid side. The blood of some mice was procured when the staining was at a maximum and of others when it was fading. The hue of the plasma colored with cresol red corresponded in general with that evident at pH 7.38 in a buffer solution, but in some individuals approached that at pH 7.55. So, too, with phenol red. It has been interesting to learn from Dr. James A. Hawkins, since the observations were completed, that he has independently obtained the same values for mouse blood by means of his accurate colorimetric procedure.[6]

[6] Hawkins, J. A., *J. Biol. Chem.*, 1924, lxi, 147.

Only the skin flaps of the mice from which blood was procured were looked at during life, but the colors there visible sufficiently indicated that the staining in these instances was typical.

The findings with cresol red and phenol red accord despite the differing ranges found for the phthaleins under controlled conditions. With both the color of the indicator in plasma from the right ventricle is such as would indicate a reaction varying from pH 7.38 to 7.55. It may be recalled that the hue of the tissues stained with cresol red, if taken at its face value, would mean that these had a pH at least as low as 7.2 and possibly less. Phenol red will indicate pH 7.2 with exactitude, at least under controlled conditions, and the connective tissue had a hue approximately corresponding thereto and the tendons one not very different; whereas the hue of all the other structures fell outside of the range of this phthalein, on the acid side, being a yellow such as is associated with pH 6.6 or less. There is good reason to believe that the findings in the blood are indicative of the actual state of affairs as concerns it. But what is one to suppose of the color manifestations within the tissues? As a first step toward answering the question tests have been made with other phthaleins.

Brom Phenol Red.

Recently Cohen has reported upon dibromo-phenol-sulfonphthalein (brom phenol red) which has, according to his brief statement, a range from pH 5.4 to 7.0. My specimen of the indicator, supplied by Hynson, Westcott and Dunning, is yellow at pH 6.47 and old rose at 7.38 when in dilute solution (0.1 per cent in a double phosphate buffer), whereas when ten times as much is present it is yellow at pH 6.24, orange at 6.64, and orange-red at 7.38. The range differs but slightly, then, from that of phenol red. 1 cc. of a 2 per cent solution is well tolerated by a 30 gm. mouse, and the staining is such that the tissue hues can be checked against the more dilute of the buffer series just mentioned. The coloration is at a maximum within 1 hour, has largely faded within another, and vanishes completely overnight without appearing to have affected the health. The animals void dark red urine.

As already remarked, the yellowish pink of the hairless surfaces of

stained mice nearly resembles that after phenol red, and a like difference between the color of the blood and tissues is demonstrable in the ears. During prolonged or heavy etherization the hue of the body surfaces changes from pink toward yellow. Clipped fragments of epidermis are always yellow, like the adipose tissue attached thereto. As with phenol red most of the tissues are yellow but the brain and nerves fail to stain. As in its case, too, the connective tissue and tendons form notable exceptions, the former being yellow-pink, as is the indicator in a buffer solution of pH 7.2 and the latter pinkish yellow. In their apparent significance the findings correspond absolutely with those obtained with the other indicators.

The results with brom cresol purple, chlor phenol red (Cohen), and indicators of more pronouncedly acid range will be detailed in a paper to follow. Suffice it to say that, in so far as they bear upon the findings here described, they are in consonance with them. Brom thymol blue cannot be employed because it is even more toxic than thymol blue. An amount sufficient to produce staining brings about an extreme general acidosis, as evidenced by the greenish yellow color of the prostrated animal, a hue which, under controlled conditions, would indicate that the blood had a reaction as acid as pH 6.2. Death ordinarily takes place within a few hours.

The Factor of Asphyxia.

Asphyxia notably influences the hue of tissues stained with the phthaleins. In mice killed after receiving cresol red there occurs within a few minutes an alteration in the color of the blood within the smaller vessels from red to yellow. The tissues have, during life, the latter hue, that is to say are apparently so acid as to be beyond the range of the indicator and, as would be expected, no change is visible in them post mortem. The same holds true for most of the tissues stained with phenol red. With this dye, however, as will be recalled, the connective tissue is a yellowy pink during life, and the body surfaces appear pink both because it underlies them, and because of the ruddy blood. Soon after death, though, the surfaces change from pink toward yellow; and the connective tissue then exposed is found to have this hue, while the blood in the smaller vessels is far less pink than during life. Analogous changes are to be noted when brom phenol red is the

stain. All may be attributed with good reason to asphyxia, which is known to make for acidity within the organs. The hue in many of the tissues of the dead animal veers toward that indicative of alkalinity when they are exposed to air. Even during life the ventilation of raw surfaces results in local changes in the reaction, as will now be shown.

Ventilation from Exposed Tissue Affects Its Reaction.

When etherized animals stained with cresol red are laid open in the absence of the protection afforded by an oil bath, the color of one of the tissues exposed *in situ* to air, but only one, namely the connective tissue, is observed gradually to change, turning from yellow to red. When phenol red has been employed, the change is rapid, indeed very rapid in subcutaneous layers stripped away from the adjacent structures; and not only is the connective tissue affected, if this most obviously, but tendons, cartilage, and bony surfaces as well. Within a few minutes all are purply red. With brom phenol red similar alterations and of the same tissues are observed. The limitation of the phenomenon in the case of cresol red falls in with the fact that its range is relatively alkaline. One may suppose that only in the case of the connective tissue, the most alkaline apparently, of the fixed constituents of the body is the further increase in alkalinity great enough to affect the indicator color.

There are many conceivable explanations for this change in reaction occurring upon exposure to air, but the most reasonable is a ventilation from the raw surfaces, carbon dioxide escaping therefrom. Since the change does not occur when the dissection is made under oil, one can be certain that altered circulatory conditions are not responsible for it. That it is not irreversible follows from the observation that everted skin flaps, containing phenol red, which have become rose-red following exposure to air, return to the original orange hue when they have been replaced against the body for a few minutes. The cause of the alterations in color was demonstrated as follows:

A mouse stained with phenol red by intraperitoneal injection was anesthetized and two symmetrical, slightly curved incisions were made through the skin of abdomen and chest, from the pelvis to the neck. Between them was left a small isthmus of skin containing the needle puncture—this in order to avoid a possible

contamination of the fields of observation with indicator solution leaking from the peritoneal cavity. Threads were tied into the upper and lower ends of the skin flaps outlined by the incisions, and the flaps themselves were rapidly separated from the underlying structures by blunt dissection with forceps and at once replaced in the original position. There was no bleeding. The animal was now placed on its back upon wet blotting paper covering a platform warmed by a water circulation; and a large glass funnel was inverted over it (Fig. 1). At one edge of the funnel rim was a notch patched with a piece of rubber dam moored

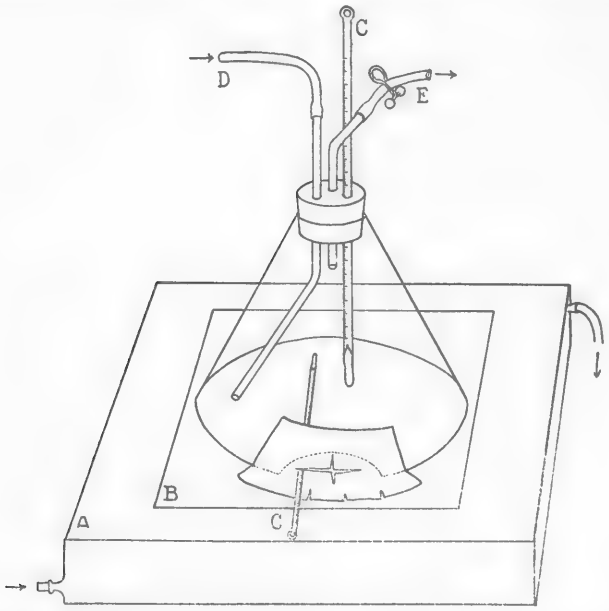

FIG. 1. *A*, metal box with circulation of warm water; *B*, wet blotting paper; *C*, thermometers; *D*, inlet for gases; *E*, outlet.

to the glass with adhesive plaster. The head of the mouse was brought to the outside through a slit in the rubber which fitted the neck snugly. Thus the animal continued to breath the room air, while the body inside the funnel could be submitted to any gas one chose to introduce. The neck of the funnel had been cut off and a three-hole rubber stopper inserted in its place. One of the holes contained the shaft of a thermometer, the bulb of which was fixed just above and to one side of the mouse. Another thermometer, passing horizontally through the rubber dam, rested on the blotting paper. A bent tube ex-

tending down almost to this latter, through the stopper, but ending well to one side of the animal, was employed to introduce the gas for test. Another tube, barely projecting into the funnel, which could be closed at will with a pinch-cock, furnished an outlet. The funnel rim fitted the blotting paper closely. The gas to be introduced was moistened and heated to body temperature by bubbling it through warm water. Little difficulty was experienced in keeping the temperature constant within the funnel, at 36–40°C., according to desire. When the animal was first put in, it was stretched on the back by means of cords attached to the legs and extending under the funnel rim. The threads from the skin flaps also passed out under the rim, but the flaps as yet lay in place.

A specimen protocol follows (see also Table I):

A female mouse of 30 gm. was given 0.4 cc. of a watery 20 per cent urethane solution, into the subcutaneous tissue of the back of the neck at 10.19 a.m., followed at 10.32 by 2 cc. of a 1 per cent solution of phenol red into the abdominal cavity. At 11.42 the anesthesia was sufficient for the operative work which was finished by 11.50. In freeing the flaps care was taken not to interfere with the supplying vessels from the groins and axillæ. There was no hemorrhage. At 11.55 the mouse was placed under the funnel, and observed until 12.08. During the 13 minutes the rate of respiration varied between 186 and 190 per minute. The temperature within the funnel was kept between 35° and 36°C. During later periods it was held close to 35°.

12.08½ to 12.13. *Respired air of an observer breathed directly into the funnel*, the lungs often being emptied more fully than usual.

12.13. *Left skin flap exposed* by traction on the threads. It appeared ruddy orange as usual, owing to the blended yellow of fat and epidermis with the pinker color of the connective tissue and blood. At 12.16 there were 190 respirations per minute, at 12.19, 182, and at 12.22 and 12.23½, 178 per minute. Respired air still breathed in until 12.24.

12.24. *Right flap pulled open and compared with left.* The latter as viewed by itself, appeared scarcely to have changed color, but it was now seen to be slightly the more ruddy of the two, indicating a change in it toward greater alkalinity, despite the exposure to expired air. The respirations had now fallen to 182.

12.24 to 12.32. Funnel repeatedly ventilated with room air and adjustment made of flaps to expose them more fully.

12.35. The flaps had become purply red. There were 182 respirations per minute.

12.39. Respirations 176 per minute. *Pure oxygen was run into the funnel* and the flow kept up until 12.51½. During this period the exposed flaps became more purple. The respirations, taken every other minute, varied between 172 and 180.

12.52. Respirations 178 per minute. *The oxygen was shut off and a flow of*

TABLE I.

Effect on the Reaction of the Ventilation Occurring from a Tissue Flap.

Time.	Period.	Procedure.	Color of exposed tissue.	Respirations per min.	Remarks.
	min.				
11.55 to 12.08	13	Mouse under funnel.	Tissue unexposed.	186 to 190	Control period. Funnel temperature 35–36°.
12.08½ " 12.13	4½	Funnel filled with respired air.	" "		
12.13		Left skin flap exposed.	Ruddy orange.		
12.13 to 12.24	11	Funnel kept full of expired air.		190 " 178	
12.24		Right skin flap exposed.	The one already bared found slightly the more ruddy.	182	
12.24 to 12.39	15	Funnel repeatedly flushed with room air.	Flaps gradually became purple.	182 to 176	
12.39 " 12.52	13	Oxygen run in.	Flaps became more purple.	172 " 180	
12.52 " 12.54	2	Oxygen shut off; CO_2 run in.	Rapid change to yellowish orange.	180	
12.54 " 1.12	18	CO_2 still run in.	Brilliant orange-yellow.	Progressive increase to 204.	Exaggerated respiration.
1.12 " 1.25	13	CO_2 shut off; oxygen run in.	Very gradual change to purple-red.	Decrease to 180.	Rapid return to normal.
		Experiment discontinued.			Funnel temperature 35°.

pure carbon dioxide started. Practically at once the color of the flaps altered toward orange. By 12.54 they had become an outspoken yellowish orange with no hint of red. The respirations were still 180 at 12.55, but as the gas continued to flow into the funnel they gradually quickened until at 1.02 there were 204 per minute. The breathing had become very exaggerated, causing the whole body to move. No signs of returning consciousness were visible. Until 1.12, when oxygen was substituted for the carbon dioxide, the breathing continued rapid and exaggerated. At 1.12 there were 196 respirations to the minute. The flaps were a brilliant orange-yellow.

1.12 to 1.25. *Oxygen run in.* The flaps were noted to have become somewhat ruddy 3½ minutes later, and the breathing to be less agitated. By 1.17 the respiratory rate had fallen to 180, and it did not alter thereafter, being 180 at 1.24. The flaps had gradually become purple-red.

The animal, in excellent condition, was sacrificed. There was no free fluid upon the raw surfaces. The phthalein which had so frequently changed color lay within the tissue.

Analysis of the phenomena just described leaves no doubt that carbon dioxide is rapidly given off from raw subcutaneous surfaces and taken up by them, with concomitant changes in the local reaction of the tissues. The observation that a raw surface submitted to expired air fails to undergo the change to ruddy purple, which takes place in room air, does not of itself suffice to show that a loss of carbon dioxide is responsible for the alteration in hue. The final proof has been supplied by exposing the surface to expired air which had been passed through soda lime to remove the dioxide. Under these circumstances the change to purple was a rapid one.

It will have been noted in the protocol that when the large skin flaps had been for some time exposed to an atmosphere of carbon dioxide—in which they soon became orange-yellow—a gradual, but eventually marked, increase in rate and amplitude of the breathing took place, and that the changes lasted so long as did exposure to the gas, disappearing after oxygen had been substituted. The quickened breathing did not occur in animals unprotected against loss of body heat, the rate then falling progressively, irrespective of the gas to which the surfaces were exposed. The question whether the hurried and gasping respiration was the manifestation of a general acidosis consequent on absorption from the exposed tissues was answered in not a few instances by a concomitant change in the color of the ears from pink toward yellow, indicating that the blood had become more acid.

When mice thus affected were removed from the funnel to an incubator, and kept at 38°C., the skin flaps having first been put back in place, many minutes elapsed before the hairless surfaces recovered the pink hue, so profound had been the disturbance of the acid-base equilibrium. The most marked instances of the sort occurred in animals given ether. In them, of course, intercurrent respiratory changes took place that were attributable to the anesthetic itself, but over and above these one could note marked and characteristic changes in the breathing as the acidosis with carbon dioxide developed. Incidentally it may be remarked that, irrespective of other possibilities and probabilities, the acidosis consequent on the anesthesia itself will suffice to explain why a protected raw surface usually appears slightly less alkaline than one exposed for some time to expired air.

The only tissue which was observed to change color during the experiments on ventilation was the connective tissue forming the raw surface of the skin flap and of the body whence the latter had been stripped. The underlying adipose tissue, muscle, and skin retained the initial yellow. In the case of the connective tissue, the intense rose-purple hue assumed on exposure to air is such as might be taken to indicate that the alkalinity had increased to pH 8.0 or more. The well known fact may be recalled in this relation that blood serum set in the air undergoes a considerable increase in alkalinity.

DISCUSSION.

However great the demerits of indicators may prove to be as gauges of local reactions within the body, the utilization of them here described has demonstrated, if only anew, facts which render their employment justifiable. Principal among these is the complexity of the conditions to be coped with if the precise pH prevailing in the interior regions of the organism during life is to be determined by other than intravital methods objective in manifestation. The least interference with blood supply, the giving of an anesthetic, the exposure of the tissue to air, postmortem changes, all alter the reaction swiftly. So, too, must many another influence, for example, trauma. A gradual recognition of these possibilities of error in the application to the tissues of the electrometric method may be traced in the litera-

ture of the subject, beginning with Michaelis' attempts[7] to determine the reaction by a procedure which involved hashing the organs in the air and extracting them with water, after cooking in some instances, and progressing to the observations of Schade and his collaborators[8] who introduced a hollow needle electrode directly into the tissues, and foresaw and made provision to meet errors incident to differences in carbon dioxide tension. Precise determinations have been reported with Schade's apparatus, but only on tissue fluids and in superficial regions.

The question of the meaning, in terms of pH, of the hues assumed by tissues stained with a given indicator must be left unanswered for the time, like that of the situation of the stain, whether only in the lymph or upon or inside of the cell, or in the intercellular substances. It will suffice at the moment to speak merely of indicator within the tissues as distinct from that within the blood. The findings with undiluted plasma more or less heavily tinted with indicator, as procured by aspiration from the heart, are readily subject to control by the micro method of Hawkins.[6] *A priori* much the same objections might be lodged against the interpretation of the tinting of the plasma in terms of pH as against a similar step in connection with the color of the tissues. One thinks immediately of protein errors and of a possible loss of carbon dioxide through the oil. But, as already remarked, Dr. Hawkins' unpublished data prove that the pH of mouse blood has essentially the range which it would appear to have from the hue on vital staining with the phthaleins as I have described it. One is justified in saying with regard to the tissue coloration merely that the evidence with different phthaleins accords remarkably in that it indicates the reaction within them all to be less alkaline than that of the blood and in most cases to be frankly acid. In a succeeding paper results with indicators suited to the determination of this presumptive acidity will be described.

It may be objected, in connection with the examination made of bits of the viscera, that the conditions of study were supravital rather than vital. The objection is valid, no matter how swiftly the bits are re-

[7] Michaelis, L., and Kramsztyk, A., *Biochem. Z.*, 1914, lxii, 180.

[8] Schade, H., Neukirch, P., and Halpert, A., *Z. ges. exp. Med.*, 1921, xxiv, 11.

moved and inspected. It will lose force only when a method has been devised whereby the reaction within highly vascular or well encapsulated organs can be discriminated while they are still *in situ*. Fortunately such a discrimination is possible in the case of many tissues; and some of them, cartilage from the tip of the tail, epidermis, connective tissue, fat, can be viewed directly in the absence of any complicating acidosis such as anesthesia might produce.

The demonstration of a relative acidity within tissues is foreshadowed by not a little previous investigation. Theoretically, as Schade remarks, every cell constitutes a focus from which acid streams out, with result that one would expect a more or less gradual decline in the hydrogen ion concentration as the products of activity of the cell pass from this latter to reach eventually the blood of the right heart. He states that the pH of the intercellular fluids of the subcutaneous tissue is definitely, if very slightly, less than that of the blood. The reaction of the perimuscular fluid of the exercised rabbit falls as low as pH 6.6. Inflammation is accompanied by a marked local acidosis of the tissue fluids; and the pus of furuncles may be acid as pH 5.96. Crozier[9] has noted that certain ciliates and some of the cells of insect tissues stain with brom thymol blue in such wise as to indicate a condition of acidity. The dye is toxic for the mouse, but seems not to be for insects. Crozier suggests that protoplasm in general may be acid.

Krogh has shown the case with which carbon dioxide passes through animal membranes;[10] and the change in reaction of raw surfaces exposed to air cannot, in view of this fact, be considered as a surprising one. But the demonstration that a general acidosis may be produced by the exposure to the gas of large raw surfaces of notably poor vascularization speaks much for its diffusibility. Were carbon dioxide the only substance involved in respiration, it is conceivable that large subcutaneous cavities with highly vascular walls, such as might readily be prepared by operation, would serve the part of lungs, if due care were taken to ventilate them. But, unfortunately for such an experiment, oxygen diffuses with relative difficulty, about thirty-two times as slowly as the dioxide.[9]

[9] Crozier, W. J., *Proc. Soc. Exp. Biol. and Med.*, 1923–24, xxi, 58.
[10] Krogh, A., *J. Physiol.*, 1918–19, lii, 391.

SUMMARY.

Mice can be vitally stained with many of the phthalein indicators. The staining is diffuse, appearing to interfere not at all with health in the case of the majority of the dyes. The color phenomena show that these retain the character of indicators. A special technique has been evolved for the determination of the hues of the various organs, which are readily modified by extraneous influences. The ability to recognize that the pH has thus been altered is a signal advantage of the indicator method.

Phthaleins of slightly alkaline range or one that trenches slightly on acidity have been employed for the work here reported. Cresol red, phenol red, and brom phenol red have proved especially useful. The observations with the three agree closely in pointing to the existence of notable differences between the reaction of the blood and that within the tissues generally.

The hue of blood plasma from the right heart is such as to suggest that its reaction lies at about pH 7.38 ordinarily, whereas that of the most alkaline of the tissues, judging from its color, the connective tissue, would appear to have a pH of 7.2 or slightly less. The tendons seem to be nearly but not quite so alkaline. The other stained tissues without exception, are of a hue which would indicate that the reaction lies beyond the range of phenol red on the acid side, that is to say is at least as acid as pH 6.6. In a subsequent paper observations which accord with these findings, carried out with indicators of frankly acid range, will be described.

On the exposure of tissues to air, without disturbance of the circulation, some of them become alkaline. In the case of connective tissue, at least, the change is a consequence of the escape of carbon dioxide. The gas passes readily in and out, exerting a practically immediate influence on the color of the tissue bared by eversion of a skin flap; and so much may be absorbed on exposure to pure carbon dioxide, when the surface is large, that a general acidosis results.

The precise interpretation of the color changes in terms of pH waits necessarily upon further work.

RESISTANCE OF BACTERIAL SPORES TO THE TRIPHENYLMETHANE DYES.

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Plate 18.

(Received for publication, November 25, 1924.)

The resistance of bacterial spores to dyes is of considerable clinical importance. Dyes are being used extensively as disinfectants in the treatment of wounds; and wounds, particularly in war time, may contain spores of pathogenic bacteria. Such spores may lie dormant for days or months.[1] The bactericidal action of dyes in the body tissues may be lost in a comparatively short time. Gentian violet, for example, ceases to be effective for more than a few hours when injected into the blood stream. How long it remains effective in open wounds or closed cavities is unknown. In this connection the fact may be significant that it does not remain bactericidal long in an alkaline solution although alkali increases its action at first.[2] Needless to say, if spores fail to be killed by a dye they may lie dormant until its bactericidal action is lost and may then develop and account for secondary infections in wounds previously thought to be sterile. It has been stated that spores are killed by certain dyes, especially by gentian violet.[3] The experiments described in the present paper indicate that spores are highly resistant to this dye.

The resistance of spores to dyes is also of theoretical importance. If spores are dye-resistant, the possibility of the development of a dye-fast strain comparable to the arsenic-fast strains of trypanosomes must be considered. It is conceivable that spores developing after most of the bacteriostatic power of a dye has been lost may acquire

[1] Burke, G. S., *J. Infect. Dis.*, 1923, xxxiii, 274.

[2] Burke, V., and Grieve, I., *Am. J. Med. Sc.*, 1924, clxviii, 98.

[3] Churchman, J. W., *J. Exp. Med.*, 1912, xvi, 221; 1921, xxxiii, 569; 1923, xxxvii, 1, 543; *J. Am. Med. Assn.*, 1922, lxxix, 1657; *J. Urol.*, 1924, xi, 1.

dye-fastness, or resistance. Experiments in the present paper indicate that vegetative cells of *Bacillus anthracis* can gain in their ability to grow in the presence of gentian violet.

EXPERIMENTAL.

Experiment 1. To Determine the Resistance of Bacterial Spores to Triphenylmethane Dyes.—The technique adopted was designed to eliminate the possibility of apparent death of spores being due to inhibition by dye carried over in transplants or to death of vegetative cells arising from developing spores.

Technique.—1 cc. of an old culture of *B. anthracis* was placed in a test-tube. 15 cc. of a saturated aqueous solution of gentian violet was added. (The saturated solution was made by adding 6 gm. of gentian violet to 30 cc. of water, warming to 80°C. for 20 minutes, and then filtering.) The dye-spore suspension was placed in the incubator at 37°C. for 24 hours and then plated out in agar in three successive dilutions by the loop method, the first tube of agar receiving a loopful of the spore-dye suspension, the second tube a loopful from the first tube, and so on. A control tube was run, with 0.85 per cent saline in place of dye. It received the same treatment except that just before plating it was heated to 80°C. for 10 minutes to eliminate vegetative cells. The plates were incubated for several days and counts made of those from the third dilutions. Twenty-five anthrax colonies appeared on the third plate of the dye-spore suspension and 375 on the third control plate.

By this procedure the spores are washed in agar to remove the dye. The agar readily absorbs the dye and since there is organic matter present tends to reduce its bactericidal action. This method gives roughly quantitative results. When the exposure to the dye lasts for 24 hours the factor of developing spores in both the dye and saline suspensions must be considered and controlled before accurate quantitative results can be obtained. The technique used is satisfactory for demonstrating the resistance of some of the spores but not for determining accurately the percentage surviving.

The experiment was repeated to confirm the initial finding that some anthrax spores at least are very resistant to gentian violet. Methyl violet gave results similar to gentian violet. This is to be expected since gentian violet is either a particular type of methyl violet or a mixture of methyl and crystal violet. Exposure to saturated gentian violet for 10 minutes at 80°C. gave comparable results

although apparently a slightly smaller percentage of the spores developed. Using *Bacillus subtilis* in place of *Bacillus anthracis* produced similar results. The spores of *Bacillus subtilis* appeared to be somewhat more resistant to gentian violet than the spores of *Bacillus anthracis* as a slightly larger percentage developed after exposure to the dye.

With some disinfectants weaker solutions are more effective than saturated solutions. It was thought that this might account for any discrepancies between the results of our work and those of others. Some spores of *Bacillus anthracis* were found to resist dilutions of gentian violet up to 1:10,000 for 24 hours at 37°C. A saturated solution has greater bactericidal action than weaker solutions.

The results obtained in this experiment indicate that not a few spores of Gram-positive bacteria are highly resistant to the triphenylmethane dyes. That they are more resistant than the vegetative cells of Gram-negative organisms such as *Bacterium coli* can readily be shown. One need only place a mixture of *Bacillus subtilis* and *Bacterium coli* in a saturated aqueous solution of gentian violet, follow the technique used in this experiment, and a pure culture of *Bacillus subtilis* will result. A relatively shorter exposure will suffice.

Experiment 2. To Determine whether a Dye-Fast Strain of Bacillus anthracis Might Develop upon Exposure to a Non-Bacteriostatic Concentration of Gentian Violet.—

Technique.—The anthrax culture used in this experiment was obtained from a third dilution plate made in an experiment like Experiment 1. This colony apparently had developed from a spore or spores resistant to gentian violet. Strokes from this culture were made on agar plates containing concentrations of gentian violet from 1:100,000 to 1:1,000,000. Extract agar containing 1 per cent dextrose and with a reaction of pH 7.5 was used. From a colony arising in the plate containing the greatest concentration of dye, strokes were made on other plates containing the same and greater concentrations of dye. This process was repeated through a number of generations.

By this method it proved possible to obtain a culture of anthrax that would grow in a concentration of dye at least twice that which inhibited the original culture. Fig. 1 shows two dye-tolerant colonies growing in a dilution of 1:400,000 gentian violet. This represents

the limits of tolerance obtained by this method from one culture. The agar in half the plate contains dye in this concentration. The agar in the other half of the plate is dye-free. The plate shows three strokes, the two lower from a dye-resistant culture and the upper stroke from the stock culture. The stock culture did not contain any organisms capable of growing in this concentration of dye in agar. The tolerance of bacteria for the dye has been found to vary with the pH.[2]

Agar containing a 1:400,000 dilution of gentian violet is distinctly colored and the colonies are also colored. A microscopic examination of the individual cells of such colonies shows the vegetative cells faintly stained. Since the colonies steadily increase in size the cells must be multiplying despite the presence in them of the dye.

The cells of a transplant of *Bacillus anthracis* as well as the strains may vary in their tolerance for gentian violet. The growth of only two colonies from the many cells stroked on the dye agar in Fig. 1 indicates cell variation in ability to tolerate dye. We believe it indicates cell variation in resistance rather than favorable spots in the agar since the colonies contain dye and strokes from these colonies on similar plates gave identical results. Their growth indicated the ability of only a few cells to start growth and produce colonies in this concentration of dye. In a somewhat weaker solution of the dye a stroke from our dye-resistant strain produced a uniform growth across the dye agar while a stroke from the stock culture failed to produce growth on the dye agar, indicating increased tolerance for many if not all the cells. The presence of dye-resistant and non-resistant strains within a culture of *Bacillus anthracis*, as demonstrated in cultures of *Bacterium coli* by Churchman, should be considered. However, the technique employed by us eliminated in the beginning any non-resistant strains that may have been present.

The colonies appearing on the dye agar grew more slowly than the controls. This delayed growth is not due to a loss of bacteriostatic power of the dye as a result of exposure to the agar. The agar was remelted and restroked with the same result as at first, the colonies that grew on the dye agar starting later and developing more slowly than the control.

Colonies growing in the greater concentrations of dye do not always

appear as typical anthrax colonies (Fig. 2). However, strokes from these atypical colonies on dye-free agar result in the development of typical colonies, and broth cultures injected into guinea pigs cause death with typical symptoms.

DISCUSSION.

The results obtained in Experiment 2 demonstrate that the individual vegetative cells of *Bacillus anthracis* vary in their tolerance for gentian violet and that tolerant strains can be obtained by exposure to non-bacteriostatic concentrations of the dye. These facts may be of clinical importance. The variation in resistance and ability to increase resistance may make possible the development of more or less dye-fast strains of *Bacillus anthracis*, and of other organisms as well, during an infection in which dye therapy is used. The organisms may increase in resistance to the dye just as in resistance to immune bodies.

Dye therapy, as at present applied, may favor the development of dye-fast strains of bacteria. Young and Hill have shown that concentrations of gentian violet greater than 1:140,000 are not well tolerated in the blood stream.[4] We have shown that *Bacillus anthracis* can develop a resistance to and grow in a dilution of 1:400,000 gentian violet in agar. We do not know the concentration of dye anthrax will resist in the blood stream. The short period during which the dye circulates in full strength, followed by weaker concentrations for some time, favors the development of dye-fast organisms. In open wounds where 1 per cent or even saturated solutions of dyes can be used there should be little opportunity for the development of a dye-fast strain of non-spore-bearing bacteria unless the opportunity is offered in the deeper tissues reached only by the diluted dye. However, spores may resist the dye and then develop after its action is greatly reduced. The resulting cells may develop dye-fastness, gain entrance to the blood stream, and resist intravenous injections. This suggests the advisability of using the maximum strength of dye in all applications with the object of killing the spores if possible and destroying all vegetative cells. Dye

[4] Young, H. H., and Hill, J. H., *J. Am. Med. Assn.*, 1924, lxxxii, 669.

applications should be frequent enough to destroy late developing spores.

The possibility of enhancing the value of dye therapy by rotating the dye treatment deserves consideration. When two dyes are effective against an organism, or organisms in general, it may be advisable, if it can be shown that dye-fast strains develop in the body, to use the first dye in full strength and if the wound or blood stream is not sterilized to use the other dye in the second application. This may prove more effective than continued application of the same dye since increased resistance to one dye may not be accompanied by increased resistance to all dyes. Rotating the dyes may be more effective than combining two dyes as suggested by Churchman.

Churchman considers it advisable when treating mixed infections to combine two dyes, one effective against Gram-positive organisms and the other effective against Gram-negative organisms. But, as the present work shows, there is ground for the belief that any dye destroying the Gram-negative organisms will also destroy the Gram-positive organisms, exclusive of spores.

The ability of bacteria to increase in tolerance for dyes has a bearing on the testing of antiseptics. Dakin and Dunham in testing the bactericidal action of various dyes in weak concentration for *Staphylococcus aureus* found that after some hours the organisms grew unchecked.[5] They concluded that, "the dyes in question are readily inactivated and that the presence of apparently unchanged coloring matter in the mixture is no evidence of the continuation of any antiseptic action." In view of our experiments it is possible that the growth of the organisms after the initial inhibition is due to an increase in tolerance for the dye rather than to a lessening in the action of the latter.

CONCLUSIONS.

1. Bacterial spores are highly resistant to the bactericidal action of the triphenylmethane dyes. Many *Bacillus anthracis* and *Bacillus*

[5] Dakin, H. D., and Dunham, F. K., A handbook of antiseptics, New York, 2nd edition, 1918, 92.

subtilis spores resist a saturated aqueous solution of gentian violet for 24 hours at 37°C. They also resist exposure to the same dye solution for 10 minutes at 80°C.

2. The selective bactericidal action of these dyes applies only to the vegetative cells. Spores of the Gram-positive bacteria are more resistant to these dyes than the vegetative cells of Gram-negative bacteria.

3. The vegetative cells of *Bacillus anthracis* show variation in resistance to the bacteriostatic action of gentian violet. The spores of *Bacillus anthracis* show variation in resistance to the bactericidal action of gentian violet.

4. *Bacillus anthracis* can increase in resistance to the bacteriostatic action of gentian violet and grow in dye dilutions inhibiting the original culture. There is the possibility of pathogenic organisms becoming dye-resistant in the body when exposed to non-bactericidal concentrations. For this reason it is advisable to use the greatest concentration of dye compatible with tissue tolerance.

5. Since the spores of pathogenic bacteria may lie dormant for longer periods than the dyes retain their bacteriostatic action in the body frequent applications of the dye should be made in preventing infections by spore-bearing bacteria.

EXPLANATION OF PLATE 18.

FIG. 1. Strokes of *B. anthracis* on a plate containing gentian violet agar and dye-free agar. The upper stroke is from the stock culture and the two lower strokes from a dye-tolerant culture.

FIG. 2. A slant of gentian violet agar with deeply stained atypical anthrax colonies on the surface.

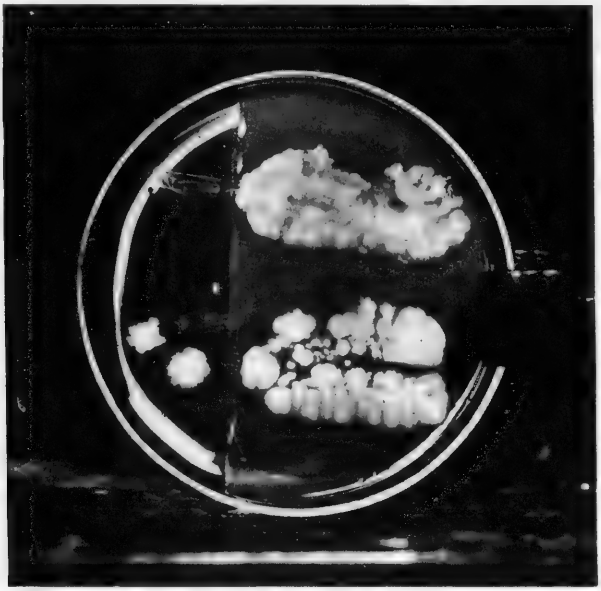

FIG. 1.

FIG. 2.

(Burke and Skinner: Triphenylmethane dyes.)

STUDIES IN EXPERIMENTAL SYPHILIS.

I. The Influence of the Size of Inoculum on the Course of Experimental Syphilis in the Rabbit.

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(Received for publication, November 19, 1924.)

INTRODUCTION.

The purpose of this communication is to record the results of an experiment which was designed to ascertain whether or not the size of the inoculum exerts any effect upon the course of experimental syphilis in the rabbit. The behavior of several syphilitic rabbits observed during the course of some other experiments had suggested the possibility that the course of this infection in rabbits, particularly the incidence and character of generalised lesions, might be dependent in part upon the number of treponemata in the inoculated material. Moreover, it seemed desirable to ascertain the influence of this factor upon the incubation period, since much stress has been laid upon the duration of the latter in attempts by several workers to differentiate various strains of *Treponema pallidum*.

The question of the influence of size of inoculum upon the course of experimental syphilis in the rabbit has received practically no attention from workers in this field. Neisser[1] concluded from his studies of experimental syphilis in monkeys that the number of treponemata in the inoculum was the most important factor in the ability of the latter to produce disease and in the speed with which a primary lesion developed. He seems to have based this conclusion upon the fact that he obtained a higher percentage of positive inoculations in apes inoculated with material from human sources containing large numbers of treponemata than in animals inoculated with material containing smaller numbers of organisms. We have not been able to find in his communications any protocols of experiments in which he used graduated doses of the same emulsion.

[1] Neisser, A., *Arb. k. Gsndhtsamte*, 1911, xxxvii, 1.

TABLE I.

Effect of Dilution of Inoculum upon Course of Experimental Syphilis.

Group.	Rabbit No.	Incubation period.	Duration of initial orchitis.	Incidence of scrotal edema.	Incidence of scrotal chancre.	Incidence of metastatic orchitis.	Generalised syphilis, exclusive of orchitis and lymphadenitis.	Lesions in order of their appearance.
		days	*days*					
A Inoculum 0.8 cc.	1	10	35	++	0	+	+	M. (45 days). E. (129 ").
	2	10	29	0	0	0	0	
	3	10	15	0	++	+	+	M. (39 "). E. (108 ").
	4	10	35	++	+	+	0	M. (45 ").
	5	10	15	++	++	0	0	
	6	10	35	++	++	+	++	" (32 "). E. (73 "), (80 ").
	Average.	10	27.3	66.6 per cent.	66.6 per cent.	66.6 per cent.	50 per cent.	
B Inoculum 0.2 cc.	7	25	34	+	0	0	0	
	8	18	27	++	++	0	+	B. (129 days).
	9	18	14	++	++	+	+++++	M. (45 "). B. (51 "), (51 "), (59 "). E. (73 "), (80 ").

	10	18	14	++	+	+	0	M. (59 days).
	11	18	33	0	++	+	++	" (51 "). B. (59 "). E. (171 ").
	Average.	17.8	24.4	80 per cent.	80 per cent.	60 per cent.	60 per cent.	
C Inoculum 0.05 cc.	12	18	21	0	+	0	++	E. (87 "), (101 ").
	13	21	38	0	+	0	0	
	14	25	14	++	++	0	0	
	15	21	24	0	+	0	+	" (87 ").
	16	13	32	0	0	0	0	
	17	21	45	0	++++	+	++++++	M. (66 "). S. (66 "). E. (80 "), (80 "). S. (94 "), (94 "), (101 ").
	Average.	19.8	29	16.6 per cent.	83.3 per cent.	16.6 per cent.	50 per cent.	

M. indicates metastatic orchitis; E., eyes; S., skin; and B., periosteum and bone.

EXPERIMENTAL.

Three groups of adult male rabbits comprising individuals of varying breeds were utilised for the experiment. The Nichols strain of *Treponema pallidum* was selected because of its high degree of virulence for the rabbit and because its behavior in this animal is better known perhaps than that of any other American strain. All inoculations were made into the right testicle and the animals were kept under observation for a period of 192 days.

The individual members of each group received the same amount of inoculum. The material used for inoculation consisted of mixed testicular emulsions from two rabbits in which the testicular lesions were comparatively fresh and were increasing in extent at the time the emulsions were prepared. The mixed emulsion contained five to ten actively motile treponemata per field and constituted, therefore, a rich inoculum. Of this emulsion, Group A, comprising six rabbits, received 0.8 cc. each, Group B of five rabbits received 0.2 cc. each, and Group C of six rabbits received 0.05 cc. each. These dilutions were selected because they represent about the limits of dosage that are ordinarily employed in this type of work. The inoculation was made into the body of the right testicle in each rabbit.

RESULTS.

For lack of space the individual protocols have been omitted and the results will be presented in tabular form. The particular points studied were (1) the incubation period, (2) the duration of the initial orchitis before there was clinical evidence of regression of the same, (3) the occurrence of scrotal edema in the inoculated testicle, (4) the occurrence of a scrotal chancre in the inoculated testicle, (5) the occurrence of orchitis or epididymitis in the uninoculated testicle, and (6) the occurrence of generalised lesions elsewhere. The incidence of these various conditions is set forth in Table I.

Analysis of Table I brings out the following facts.

Incubation Period.—The average incubation period of the group receiving the largest inoculum was least, while that of the group receiving the smallest inoculum was greatest. While there were a few exceptions to this rule they were not sufficient to modify it appreciably. Under the conditions of the experiment there was an inverse ratio between size of inoculum and incubation period, as one might expect. The difference in the average incubation period between Groups A and B was 5.8 days, that between Groups B and C, 4 days, and that between Groups A and C, 9.8 days.

Duration of Initial Orchitis.—The average duration of the initial orchitis reckoned from the appearance of the first clinical sign of infection until there was definite clinical evidence of beginning regression of the lesion did not vary appreciably in the various groups, nor was there any relationship observable between this factor and that of size of inoculum. The determination of the exact time at which regression of a syphilitic orchitis sets in is, at best, somewhat uncertain, and such differences as were encountered in this respect in the three groups would come well within the experimental error. The extremes of duration of the initial orchitis were encountered in each of the three groups.

Scrotal Edema.—This type of reaction was encountered in the majority of the animals in the two groups receiving the largest inocula. It was encountered only once in the group receiving the smallest inoculum.

Scrotal Chancres.—The incidence of scrotal chancres on the inoculated side was essentially the same in all three groups. In one animal receiving the smallest size inoculum two chancres developed simultaneously.

Metastatic Orchitis and Epididymitis.—The occurrence of orchitis or epididymitis in the uninoculated testicle was much less in the group receiving the smallest inoculum than in the other two groups, the incidence being once in six animals, or 16.6 per cent.

Metastatic Skin, Bone, and Eye Lesions.—The percentage of animals showing metastatic lesions other than orchitis or epididymitis was greatest in the group receiving the largest inoculum (66.6 per cent) and least in the group receiving the smallest inoculum (50 per cent). The difference between these two groups amounted to only 16.6 per cent, however. It should be noted at this point that the animal which showed the greatest number of metastatic lesions in the entire series was one which received the smallest inoculum. This animal, No. 17, presented the appearance of malignant syphilis, having at one time no less than four skin lesions, bilateral keratitis, and five separate scrotal chancres, two on the inoculated side and three on the opposite side.

This experiment may be said not to have fulfilled all of the expectations that were in mind at the time it was planned, since, as has

already been stated, the behavior of several animals observed in the past had led us to believe that perhaps the size of the inoculum might exert some influence upon the subsequent course of the disease. On the whole, it was impossible to obtain any clear-cut evidence that the character of the disease was affected by the dosage of inoculum. It is true that the incidence of scrotal edema was considerably less in the group of animals receiving the smallest inoculum, and the initial reaction on that account was not so impressive as in the other groups, nor was the incidence of metastatic orchitis so great. However, these factors constitute only a portion of the picture of experimental syphilis in the rabbit and should not in our opinion receive undue consideration.

There was an appreciable and regular difference in the incubation period dependent upon the size of the inoculum. As one would expect *a priori*, the incubation period was shortest in the group receiving the largest inoculum and became progressively longer as the dosage of infecting material was diminished. In this respect the experiment bears out conclusively the views of Neisser regarding the influence of quantity of inoculum upon the duration of the incubation period in experimental syphilis in apes.

On the whole, the impression which was gained from this study was that while it is possible, by increasing the number of treponemata inoculated in the testicle, to speed up the initial reaction and render it more impressive by the occurrence of a massive scrotal edema, still the general picture of the disease is but little altered by varying the size of the inoculum within the limits utilised in the experiment. It must be borne in mind, however, that even the smallest inoculum which was used was a comparatively rich one, and the strain was one which is highly pathogenic for rabbits. It is entirely possible that with less virulent strains or with even smaller inocula of a virulent strain greater differences in the course of the experimental disease might be brought out.

The behavior of one rabbit, in which the disease ran a particularly malignant course after a minimal amount of virus was inoculated, illustrates the principle that malignant syphilis is the expression of an unusual degree of susceptibility of the host to the syphilitic virus rather than an indication of exalted virulence or maximal amount of inoculum.

CONCLUSIONS.

1. The size of the dose of the infecting material exerts an appreciable effect upon the duration of the incubation period in experimental syphilis of the rabbit. Other things being equal, the larger the dose the shorter is the incubation period.

2. The size of the inoculum was not found to exercise any marked effect upon the course of the disease as a whole.

3. In individual rabbits inoculated with minimal amounts of virus the disease may run a malignant course.

STUDIES IN EXPERIMENTAL SYPHILIS.

II. The Influence of a Non-Specific Inflammatory Reaction upon the Development of the Chancre.

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Plates 19 and 20.

(Received for publication, November 19, 1924.)

INTRODUCTION.

In considering the factors that govern the initiation of syphilitic infection, whether in man or in animals, most of the emphasis has in the past been placed upon the occurrence or non-occurrence of mechanical defects of the skin or mucous membranes. There has been considerable discussion as to the necessity of a break in the continuity of the surface epithelium of these structures to permit *Treponema pallidum* to gain entrance to the body of the host. Experimental work in this field has demonstrated conclusively that in the case of the rabbit virulent strains of *Treponema pallidum* can produce a characteristic lesion on a mucous membrane surface which is intact so far as can be seen from macroscopic examination.[1,2] Furthermore, such invasion may take place without the development of a characteristic local lesion at the site of inoculation.[2]

Other factors which might conceivably influence the initiation of syphilitic infection have received attention, as, for example, the comparative availability of various sites or tissues. In this respect it has been shown that some tissues (testicle, cornea) are more favorable for inoculation than others (subcutaneous tissue), and that in a given tissue the mode of inoculation may, under conditions not

[1] Reasoner, M. A., *Am. J. Syph.*, 1917, i, 478.

[2] Brown, W. H., and Pearce, L., *J. Exp. Med.*, 1924, xxxix, 645.

perhaps clearly understood as yet, influence appreciably the course of the primary syphilitic manifestation. Thus, in a given series of rabbits inoculated intracutaneously with equal amounts of the same virus, some animals will develop a characteristic lesion at the site of inoculation and the organisms will be disseminated throughout the body, whereas other animals will develop only slight lesions or even none at all, and still infection and dissemination will have taken place.[3] It is clear, therefore, that in speaking of the initiation of syphilitic infection the distinction must be made between the occurrence or non-occurrence of a chancre at the portal of entry and the occurrence of infection, since the two have not been proved to be mutually dependent.

It seemed to us that if it were possible to obtain any knowledge of the factors which operate to suppress the occurrence of a lesion at the portal of entry, a better understanding might be had of the mechanism of resistance to syphilitic infection in general. It seemed worth while to approach this problem by seeking to determine the influence of a non-specific inflammatory reaction upon the initiation of syphilitic disease. This particular question has not, so far as we have been able to ascertain, received any attention from workers in this field.

There is some evidence to show that under certain conditions a non-specific inflammatory reaction involving the skin and subcutaneous tissues may constitute a focus which is highly resistant to infection with some types of bacteria, while, on the other hand, it may constitute a focus which is distinctly favorable to infection with other types of disease-producing agents. Some unpublished studies by one of us (Chesney) on the bacterial flora of healing wounds in human beings which had not been treated with Dakin's solution and healing experimental wounds in rabbits suggested that a healing wound is not a particularly favorable site for the growth of the ordinary microorganisms contaminating the skin. On the other hand, the work of Teague and Goodpasture,[4] who succeeded in producing typical lesions of herpes zoster upon the skin of guinea pigs which was already the seat of an acute dermatitis produced by coal tar, indicates that skin which is the seat of an inflammatory reaction may constitute a very favorable site for the establishment of infection with the virus of herpes zoster.

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[3] Chesney, A. M., *J. Exp. Med.*, 1923, xxxviii, 627.

[4] Teague, O., and Goodpasture, E. W., *J. Med. Research*, 1923–24, xliv, 185.

In the absence of any information as to what might be the case in syphilitic infection, experiments were designed to test the availability of tissues already the seat of a non-specific inflammatory reaction for the initiation and development of the primary manifestation of syphilitic infection. These experiments, as will be seen later, all tended to show that an already existing non-specific inflammatory process favors rather than impedes the establishment of the experimental infection.

EXPERIMENTAL.

Experiment 1.—Two groups of adult male rabbits of various breeds were used. In the first group (Group A), comprising five animals, a circular area of skin on the back, roughly 4 cm. in diameter, was excised under ether anesthesia, exposing the subcutaneous tissue. These wounds were allowed to heal under natural conditions, no dressings or antiseptics being applied. Crusts of inspissated serum rapidly formed, beneath which granulation tissue developed. On the 11th day after operation, when the wounds were healing and the epithelium was closing in from the sides, a second group, comprising six animals (Group B), was subjected to a similar operation. 48 hours later the superficial crusts were removed from the wounds in both groups of animals, care being taken to disturb the underlying surface as little as possible. The exposed area was then inoculated with a testicular emulsion in physiological salt solution, rich in actively motile spirochetes. The Nichols strain was used in all the experiments. A drop of the emulsion was allowed to fall on the exposed surface and was distributed evenly by gently rubbing for 1 minute. Thus, the experiment offered an opportunity of comparing the behavior, toward syphilitic infection, of a granulating wound 13 days old with that of a comparatively fresh wound (48 hours old).

All the wounds healed without at first any sign of syphilitic infection. The older wounds naturally were completely healed before the more recent ones. It was noticed that there was an increased growth of hair along the edges of the wound and along the scar when the epithelium had entirely covered the granulating surface. Shortly after the wounds had healed, clinical evidence of syphilitic infection began to be manifest, at first in the old wounds, later in the recent wounds, by the appearance of induration along the line of the wound. This induration increased rapidly in extent in both planes as a rule, so that an elliptical elevated area of induration was soon formed (Fig. 1). At the same time necrosis of the epithelium in the center of the indurated area set in and increased in extent until ultimately a remarkably large chancre was formed (Figs. 2 and 3). Characteristic examples of *Treponema pallidum* could be demonstrated with ease in the serum expressed from beneath the crust of such lesions.

The incidence of positive inoculation results in the group of rabbits with old

(13 day) wounds was 100 per cent, while that in the rabbits with fresh wounds (48 hours) was 66⅔ per cent. A distinct shortening of the incubation period was also observed in the case of the former group. These results are brought out in Table I.

Table I shows that in the group of rabbits with old wounds the incidence of positive inoculations obtained was 100 per cent and the average incubation period 25.4 days, while in the group in which a comparatively fresh wound was inoculated the incidence of positive

TABLE I.

Behavior of Recent and Older Wounds to Syphilitic Infection.

	Rabbit No.	Result.	Incubation period.	Results of node transfer.
			days	
Group A (13 day wounds).	1	Chancre.	25	Not done.
	2	"	30	Positive.
	3	"	25	Not done.
	4	"	25	Positive.
	5	"	22	Not done.
Average			25.4	
Group B (48 hr. wounds).	6	Chancre.	43	Not done.
	7	"	35	" "
	8	Negative.		Negative.
	9	Chancre.	33	Not done.
	10	Negative.		Negative.
	11	Chancre.	37	Positive.
Average			37	

inoculations was 66 per cent and the average incubation period was 37 days. In the former group the lesion on the whole attained a greater size than in the latter group, although there were exceptions to this rule.

In order to determine whether or not the two animals with recent wounds which failed to show any evidence of a syphilitic lesion at the portal of entry were in reality infected, popliteal node transfers were made to normal animals with negative results. Similar transfers from animals in each group that showed characteristic chancres were all positive, indicating that when chancres are produced on the back

in the manner described above, generalisation of the virus takes place as it does in animals inoculated on the genitalia.

The experiment seemed to indicate that a non-specific inflammatory reaction favored the initiation of syphilitic infection and, further, that a granulating wound constitutes a somewhat more favorable site for the production of a characteristic chancre than does a comparatively fresh wound. It seemed important to repeat this experiment in order to determine whether this relationship was constant, and, accordingly, a second experiment was planned which was

TABLE II.

Behavior of Recent and Older Wounds to Syphilitic Infection.

	Rabbit No.	Result.	Incubation period.
			days
Group A (16 day wounds).	12	Chancre.	28
	13	"	30
	14	"	28
	15	"	30
	16	"	28
	17	"	33
Average			29.5
Group B (48 hr. wounds).	18	Chancre.	36
	19	"	39
	20	"	44
	21	"	40
	22	Negative.	
	23	Chancre.	39
Average			39.6

similar in every respect to the first, except that the old wounds were 16 days old at the time inoculation was carried out instead of 13 days, as in the previous experiment. The results of this experiment were quite similar to those obtained in the first, and are shown in Table II.

Table II shows that in the group of rabbits whose wounds were 16 days old when inoculation was performed there were positive results in every instance, whereas in the group whose wounds were 48 hours old at the time they were inoculated there were positive takes in five out of six animals, or 83.3 per cent. Moreover, the appearance of the

lesions was again delayed in the group of animals with 48 hour wounds by an average of 10.1 days.

This experiment, while perhaps a little less striking in its results than the first, confirmed it and thus seemed to establish the fact that a granulating wound 13 to 16 days old is more favorable for the initiation and development of a characteristic syphilitic lesion than is a wound 48 hours old. This greater suitability of a granulating wound for the production of a syphilitic lesion is evidenced by (*a*) the greater number of positive takes, (*b*) the diminished incubation period, and, possibly, (*c*) the greater size of the lesion on an average.

Histological examination of the tissues involved in these 48 hour and 13 day wounds as yet uninoculated showed the following characteristics.

48 Hour Wound.—Most of the corium has been removed by the operation exposing the subcutaneous tissue. The raw surface of the wound is made up of more or less homogeneous pink-staining material in which the remains of dead nuclei are visible and comparatively few apparently living nuclei are seen. It will be remembered that prior to inoculation the crust of the wound was removed, hence, little or no fibrin is to be seen on the cut surface. Beneath this layer of necrotic tissue there is a zone composed of densely packed polymorphonuclear leucocytes for the most part, together with some large mononuclear wandering cells and lymphocytes. This layer is sharply delimited and at some points attains a thickness of almost 0.5 cm. It extends in a continuous sheet from one margin of the wound to another. Beneath this leucocyte wall the subcutaneous tissue is infiltrated with polymorphonuclear leucocytes and large mononuclear wandering cells. There are relatively few lymphocytes in this zone. The blood vessels which are most numerous at the base of the layer of subcutaneous tissue, just above the muscularis, are dilated and engorged with red cells. Beyond the wound, where the epithelium and corium are intact, the cellular reaction is not to be seen, the tissue in this area appearing normal. The epithelium at the edge of the wound is beginning to show hyperplasia.

The section shows the characteristic appearance of an early stage of inflammatory reaction in which the predominating feature is the response of the wandering cells to the stimulus produced by the injury. As yet the reparative features are in the background.

13 Day Wound.—The section of a wound such as was produced in the experiments cited above, when it has progressed 13 to 16 days toward healing is characterised chiefly by the presence of a thick layer of granulation tissue, extending

from the muscularis to the level of the intact epithelium. This layer is as much as 5 mm. in thickness in some parts. The surface of the wound is made up of a layer of fibrin containing polymorphonuclear leucocytes, large mononuclear wandering cells, and lymphocytes, although the first named predominate by far. Beneath this leucocyte-fibrin layer there are enormous numbers of fibroblasts showing a parallel arrangement for the most part, their nuclei large, pale-staining, and elongated, with prominent nucleoli. Polymorphonuclear leucocytes and lymphocytes are relatively few in this area. Many newly formed capillary loops are visible in this zone and the blood vessels at the base and margins of the granulating area are engorged. There is a striking hyperplasia of the epithelium at the margins of the wound. Here the epithelium is many times its normal thickness and its extension over the granulation tissue is beautifully illustrated.

In brief, the section of the 13 day wound shows the characteristic features of an inflammatory reaction which has progressed to the point where the processes of repair constitute the predominant feature.

It was apparent from a study of the histological picture of the two types of wounds that the cellular picture was quite different, as one might expect. In the 48 hour wounds the picture was predominantly that of reaction on the part of existing blood vessels and wandering cells, with little or no participation as yet by the reparative processes. In the 13 day wounds, on the other hand, the picture was predominantly that of activity of the reparative processes.

In view of the manifest difference in the relation of early and late inflammatory reactions to a superimposed syphilitic infection it seemed desirable to produce a series of inflammatory reactions of varying degree of severity upon the skin of rabbits in order to determine what type of reaction or what stage in the reaction is most favorable to the establishment of infection. The protocol of the experiment follows.

Experiment 3.—Six groups of adult male rabbits of various breeds were utilised. Care was taken to distribute the representatives of the various breeds, as far as possible, equally throughout the groups. For the most part the ordinary grey and brown rabbits were used.

Inoculation.—The site of inoculation was always the same—the lower dorsal and upper lumbar regions. The inoculum was in each instance the same and consisted of 0.05 cc. mixed testicular emulsion in physiological salt solution from two rabbits with actively developing orchitis. The inoculum contained numerous actively motile treponemata per field and was regarded as very rich

in these organisms. It was allowed to drop on the exposed surface and was then gently spread with the gloved finger.

Group A. Four Males. Control.—No inflammatory reaction. In this group the hair on the back was clipped but nothing was done to the skin proper. The inoculum was rubbed directly upon the macroscopically unbroken skin.

Group B. Four Males. Control Subcutaneous Inoculation.—No preparation other than clipping the hair. The bulk of the inoculum was deposited about 2 cm. from the point where the needle penetrated the skin.

Group C. Four Males. Scarification.—The animals were anesthetised with ether, the hair clipped from the back, and an area of epidermis shaved off by means of a razor, in much the same manner as when Thiersch skin grafts are cut. The exposed surface bled rather freely and some exudation of serum had occurred before the inoculations were made.

Group D. Seven Males. Preliminary Coal Tar Dermatitis.—An acute dermatitis was produced by three preliminary applications of coal tar to the hair-clipped skin of the back at 48 hour intervals. 48 hours after the last application, the animal was anesthetised and the inspissated tar was removed with forceps, exposing a raw, hyperemic surface. In peeling off the inspissated tar the epidermis was noted to be thicker than normal and some of the superficial layers of the epidermis appeared to have been removed.

Group E. Fresh Wounds. Six Males.—Under ether anesthesia an elliptical area of skin was excised, exposing the subcutaneous tissue. The excision was carried out 1 to 2 hours before the inoculation. The wounds were moistened with a few drops of saline solution just prior to inoculation.

Group F. Old Wounds. Six Males.—Under ether anesthesia an elliptical area of skin was excised 14 days prior to the inoculation. No attempt was made to keep these wounds sterile. Just before inoculation the crusts were removed with sterile forceps and swab cultures made of the granulating surface. An abscess had developed in one of the wounds, the others appeared clean. Cultures were made aerobically on rabbit blood agar plates. They were all sterile except the one from the abscess which yielded a Gram-positive coccus that grew in chains.

The rabbits were kept under observation for a period of 90 days. The results are shown in Table III.

It will be seen from Table III that no animal in Group A, in which the unbroken skin was inoculated, developed lesions. In Group B, which received subcutaneous inoculations, no lesions developed in two animals, whereas in the other two characteristic syphilitic lesions made their appearance. It is of interest to note that in both of these latter a lesion made its appearance at the point where the needle penetrated the skin, and in only one did a lesion appear in the sub-

TABLE III.

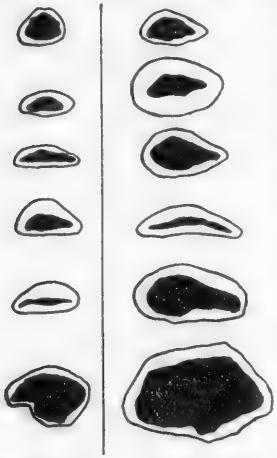
Relation of Inflammatory Reactions of Various Types and Age to a Superimposed Syphilitic Infection.

	Rabbit No.	Syphilitic lesion.	Incubation period.
			days
Group A (unbroken skin).	24	None.*	
	25	" *	
	26	"	
	27	"	
Group B (subcutaneous inoculation).	28	None.*	
	29	Two chancres.	34
	30	None.†	
	31	Chancre.	34
Group C (shaved skin).	32	Chancre.	30
	33	"	26
	34	"	34
	35	"	26
Average			29
Group D (preliminary coal tar dermatitis).	36	Numerous papules.	34
	37	" "	34
	38	Few "	34
	39	Numerous "	38
	40	Several "	34
	41	" "	34
	42	Numerous "	34
Average			34.5
Group E (fresh wounds).	43	Chancre.	34
	44	"	38
	45	"	38
	46	"	30
	47	"	38
	48	"	38
Average			36
Group F (14 day wounds).	49	Chancre.	30
	50	"	22
	51	"	26
	52	"	26
	53	"	21
	54	"	20
Average			24

* Lymph node transfer to normal animals was performed and in each instance was negative.

† Lymph node transfer to normal animals was positive.

cutaneous tissue where the bulk of the inoculum was deposited. In one of the animals in this group in which no local lesion developed, infection, nevertheless, occurred as shown by positive lymph node inoculation in normal rabbits. In all of the animals (Group C) in which a scarified area was inoculated characteristic chancres developed. In the case of the animals with fresh wounds (Group E) chancres developed ultimately in every instance, the average incubation period being 36 days, and in the animals with 14 day wounds (Group F) chancres also developed in each instance, the average incubation period in this group being 24 days. In the latter group the maximum size attained by these chancres was appreciably greater than that attained by the chancres developing in the fresh wounds, as is shown in Text-fig. 1. In the case of the animals in which a dermatitis was produced prior to inoculation by the application of coal tar (Group D), typical syphilitic lesions resulted in each instance. In many of them the lesions were exceedingly numerous and were distributed over the entire inoculated area. They were small, indurated papules, violet-red in color, at first discrete but later tending to coalesce and undergo superficial necrosis (Figs. 4 and 5).



Group E (fresh wounds). Group F (14 day wounds).

TEXT-FIG. 1. Experiment 3. Tracing of chancres of rabbits, taken at time of maximum size, showing greater size attained by those developing in old wounds (Group F) as contrasted with those developing in fresh wounds (Group E). The solid black area indicates the exact outline of the area of necrosis. The black line indicates the outer limit of the zone of induration. Reduced to 1/3 original size.

Coal tar applied to the skin of a rabbit in the manner outlined produces, as is well known, a dermatitis in which a prominent feature of the reaction is the proliferation of the epithelial cells of the skin. In sections from such an area there is to be seen marked thickening of the epidermis occasioned by a remarkable hyperplasia of the basal cells. One sees down growths

of epithelium and proliferation of cells lining the hair follicles. There are some edema and dilatation of the capillaries and an increase in the wandering cells and fixed tissue cells, but the most striking feature is the growth of epithelial cells.

DISCUSSION.

The experiments would indicate that a non-specific inflammatory reaction involving the skin and subcutaneous tissue favors the initiation and development of a primary syphilitic lesion in the rabbit. Even comparatively slight injuries to the skin, such as are produced by shaving off the epithelium, constitute a favorable nidus for the survival and multiplication of *Treponema pallidum* when applied in large numbers. It is, of course, impossible to determine the extent to which the injury alone is responsible for the establishment of syphilitic infection, as opposed to the part played by the local inflammatory reaction, since with every injury there occurs a greater or less reaction upon the part of the body. We are inclined to attribute considerable importance to the latter factor, since in general our experiments have shown that the more advanced the non-specific inflammatory reaction at the time of inoculation, the sooner and the more regularly does the primary syphilitic lesion appear and the greater size does it ultimately attain. The most favorable time for inoculation of a wound was found to be that when the wound was granulating nicely and was well on the way toward healing.

The reason for the apparently greater susceptibility of granulation tissue to syphilitic infection is not entirely clear. One explanation which should be considered is the possibility that phagocytosis is less extensive in a granulating wound than in a fresh one.

Not much is known concerning the degree to which phagocytosis operates in syphilitic lesions. Ehrmann[5] claims to have found histological evidence in chancres of phagocytosis of treponemata by the large wandering cells. Levaditi[6] has seen in sections of the lungs of congenitally syphilitic children what he regards as instances of phagocytosis of treponemata by the macrophages and polymorphonuclear leucocytes. On the other hand, Zinsser[7] as a result of his experiments

[5] Ehrmann, S., *Wien. klin. Woch.*, 1906, xix, 828.

[6] Levaditi, C., *Ann. Inst. Pasteur*, 1906, xx, 41.

[7] Zinsser, H., Infection and resistance, New York, 2nd edition revised, 1918, 515.

with mice is very doubtful if phagocytosis of treponemata actually occurs before the death of the organisms themselves. If phagocytosis of treponemata by the macrophages is a prominent factor in reaction to syphilitic infection and phagocytosis by the polymorphonuclear leucocytes is rare, then we might expect it to be less common in the fresh wounds in which the latter cells predominate, and hence we should expect the greatest number of takes in fresh wounds, which is the reverse of what has been obtained in our experiments. It would appear that before phagocytosis can be invoked as the sole explanation for the results we have obtained, much more information as to the extent to which it occurs in syphilitic infection and the type of cell which participates in the process must be forthcoming.

Some observations of Levaditi and Yamanouchi[8] have a bearing perhaps upon the phenomenon which we have described, and should be mentioned at this point. These investigators inoculated the anterior chamber of the eyes of a series of rabbits with fragments of syphilitic corneas and studied the eyes at varying intervals after inoculation to determine their treponemal content. They found that the treponemata in the inoculated fragment rapidly degenerated for the most part and there was no evidence of immediate multiplication of the organisms in the aqueous humor. Later, when organisation had set in around the inoculated fragment the organisms were found to be exceedingly numerous in the new tissue surrounding the fragment, and particularly in those regions in which there was regeneration of epithelium. These authors are convinced that the treponemata do not begin to multiply as soon as they are inoculated but that growth is retarded until the inflammatory reaction provides an abundant food supply. They would attribute the long incubation period in syphilis to delayed growth of the treponemata occasioned by lack of suitable nutritive material. It is evident that one might just as readily explain their results by assuming that the inflammatory reaction is the product of the growth of treponemata and is delayed by reason of the initial retardation of growth of these organisms. They did not perform the crucial experiment of inoculating a region which was already the site of an inflammatory process. Nevertheless, the phenomenon observed by them appears to be analogous to that which we have described, and it is possible that the explanation they offer may serve to explain both phenomena. In other words, the apparent greater

[8] Levaditi, C., and Yamanouchi, T., *Compt. rend. Soc. biol.*, 1908, lxiv, 313.

susceptibility of granulating wounds to syphilitic infection may be due to the fact that the non-specific inflammatory reaction acts to provide an increased food supply for the treponemata.

Histological study of such wounds has seemed to us to bear out such a possibility. In granulating wounds, as is well known, the reparative processes predominate, and we have been struck by the evidence of cell growth that one sees in sections of such wounds. Large numbers of new fibroblasts are being formed and there is marked hyperplasia of the basal cells of the epithelium at the wound margins. It is evident that conditions are extremely favorable to cell growth. Not only may substances be present which act as a stimulus to the growth of the fixed connective tissue cells ("trephones" of Carrel,[9] "archusia" of Burrows[10]), but in addition there must be an abundance of foodstuffs necessary to the new cell formation. It is possible that the same factors which bring about or contribute to active cell proliferation in a wound may also exert a favorable effect upon the growth of the treponemata. We do not wish to concern ourselves at this point with the nature of these factors; whether they represent hypothetical growth-promoting substances derived from wandering cells, or an excess of nutritive substances from the blood, or metabolic products of cell nutrition, is beside the point just now. We wish merely to point out that there may be a similarity in the response of both body cells and *Treponema pallidum* to the factors which stimulate cell growth in healing wounds; in other words, that anything which favors the growth of the former may also favor the survival and growth of the latter.

Such an hypothesis suggests the possibility of a close agreement between the metabolism of the human cell and that of *Treponema pallidum*. Theobald Smith[11] has already suggested such a possibility, but nothing definite is known about the metabolism of *Treponema pallidum*. Its nutritional requirements are as yet very imperfectly understood. It is known to be a fastidious organism, one that will not grow upon the ordinary laboratory media, yet will survive for decades within the animal body. Despite the close proximity to

[9] Carrel, A., *J. Am. Med. Assn.*, 1924, lxxxii, 255.
[10] Burrows, M. T., *J. Med. Research*, 1923–24, xliv, 615.
[11] Smith, T., *J. Am. Med. Assn.*, 1913, lx, 1591.

living cells it finds sufficient food to maintain life and insure pathogenicity. From this well known fact it is but a step to the assumption that some of the factors which operate in the human body to bring about rapid cell growth may exert a favorable effect upon the growth of the treponemata.

As a corollary to this hypothesis those portions of the body where there is active cell growth should be particularly favorable sites for the growth of *Treponema pallidum*. In this connection it may be pointed out that the testicle is a particularly favorable site for inoculation with these microorganisms and there is probably no portion of the body in which there is a greater formation of new cells.

The recent work on vaccine virus and its relation to actively growing cells of ectodermal origin is worthy of mention at this point, because of its possible bearing upon the hypothesis that we have offered.

Levaditi and Nicolau[12] showed that by simple epilation and subsequent intravenous inoculation of a rabbit with vaccine virus, it was possible to bring about localisation of the virus in the hair follicles from which the hairs had been removed. These investigators found, furthermore, that within 24 hours after the removal of the hair in the normal animal there was histological evidence of proliferation of the epithelial cells at the base of the hair follicle, with frequent mitotic figures. This process lasts 3 to 4 days. In the infected animal the same proliferation of epithelial cells at the base of the hair follicle is seen at the end of 24 hours, but the proliferative process lasts longer than in the normal animal and sections made at a later time show that the area where there is active proliferation of epithelial cells is also the site of characteristic changes produced by the vaccine virus.

Levaditi and Nicolau[13] also found that when epitheliomas of rats and mice are inoculated directly with vaccine virus the virus establishes itself in the tumor cells, brings about an increase in size of the tumor, and apparently vegetates and even multiplies there. Furthermore, if the virus is inoculated intravenously in rats or mice with epitheliomas it localises in the tumor cells. Similar relations were not found to hold for sarcomas, however. On the basis of these observations it would appear that vaccine virus has a particular affinity for actively growing cells of ectodermal origin. It may not be amiss to point out that vaccine virus and *Treponema pallidum* are alike in one respect; namely, their ability to multiply in the testes of suitable animals. It is possible that both may also possess a particular affinity for actively growing cells of ectodermal origin.

[12] Levaditi, C., and Nicolau, S., *Compt. rend. Soc. biol.*, 1922, lxxxvi, 986.

[13] Levaditi, C., and Nicolau, S., *Ann. Inst. Pasteur*, 1923, xxxvii, 443.

The hypothesis that we have offered for the phenomenon which we have described, namely that active proliferation on the part of certain cells of the body, particularly the fixed connective tissue cells and the epithelial cells, is directly associated with multiplication of *Treponema pallidum*, may be invoked to explain certain phenomena observed in the course of syphilitic infection in human beings. It may explain why treponemata are so extremely numerous in the human syphilitic fetus, since an enormous cellular proliferation is taking place in the latter. Furthermore, it is conceivable that the phenomenon which we have described may be related to the well known fact that trauma in a syphilitic individual predisposes to the development of a syphilitic lesion at the site of injury.

Whatever the explanation of the phenomenon, there can be no doubt that a non-specific inflammatory reaction in the rabbit furnishes a particularly favorable site for the survival and growth of virulent strains of *Treponema pallidum;* and for the development of a characteristic chancre, granulating wounds are especially favorable. At times more than one chancre may develop in such a wound (Fig. 6) and it is even possible to produce chancres which in their form correspond to a predetermined pattern (Fig. 7). The finding discloses a new method for producing syphilis in the rabbit that has certain manifest advantages from an experimental standpoint. It would be of interest to determine to what extent this method may be applied to the problem of transmission of other infectious diseases to laboratory animals.

SUMMARY.

Rabbits can be successfully infected with syphilis by the inoculation of surface wounds with virulent strains of *Treponema pallidum*. Old granulating wounds in these animals constitute a particularly favorable terrain for syphilis inoculation, the chancre developing relatively soon and attaining a greater size than the lesion occurring on the basis of a fresh wound. Coal tar dermatitis renders the skin susceptible to infection with *Treponema pallidum*.

The possible reasons for these phenomena are discussed and an hypothesis to account for them is advanced.

EXPLANATION OF PLATES.

PLATE 19.

FIG. 1. Side view of beginning of chancre developing in 16 day wounds, taken 27 days after inoculation and 5 days after appearance of lesion.

FIGS. 2 and 3. Same lesion 25 days later.

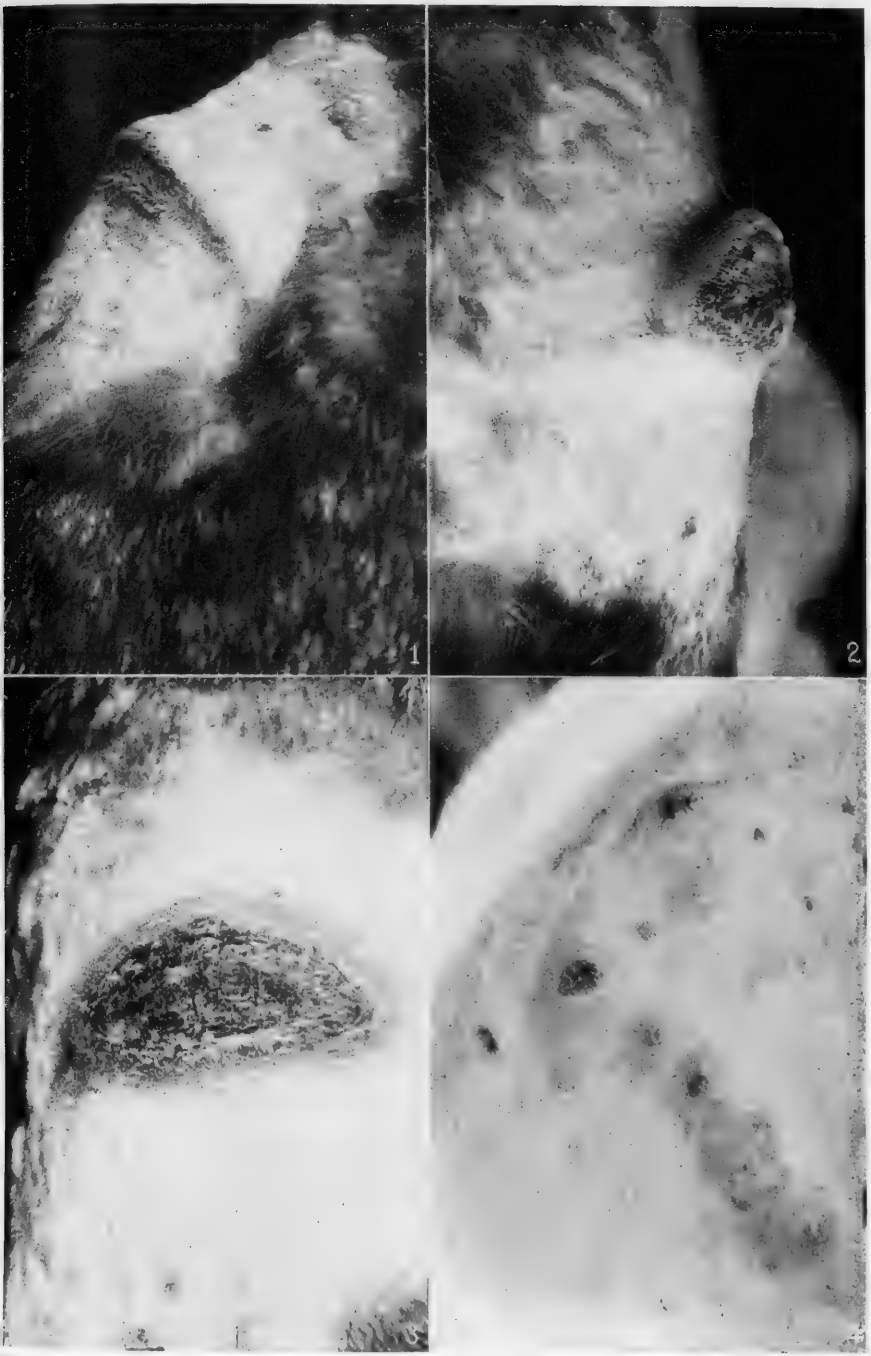
FIG. 4. Syphilitic papules produced by inoculating skin which was previously the seat of a dermatitis produced by coal tar. Taken 48 days after inoculation and 14 days after the appearance of lesion.

PLATE 20.

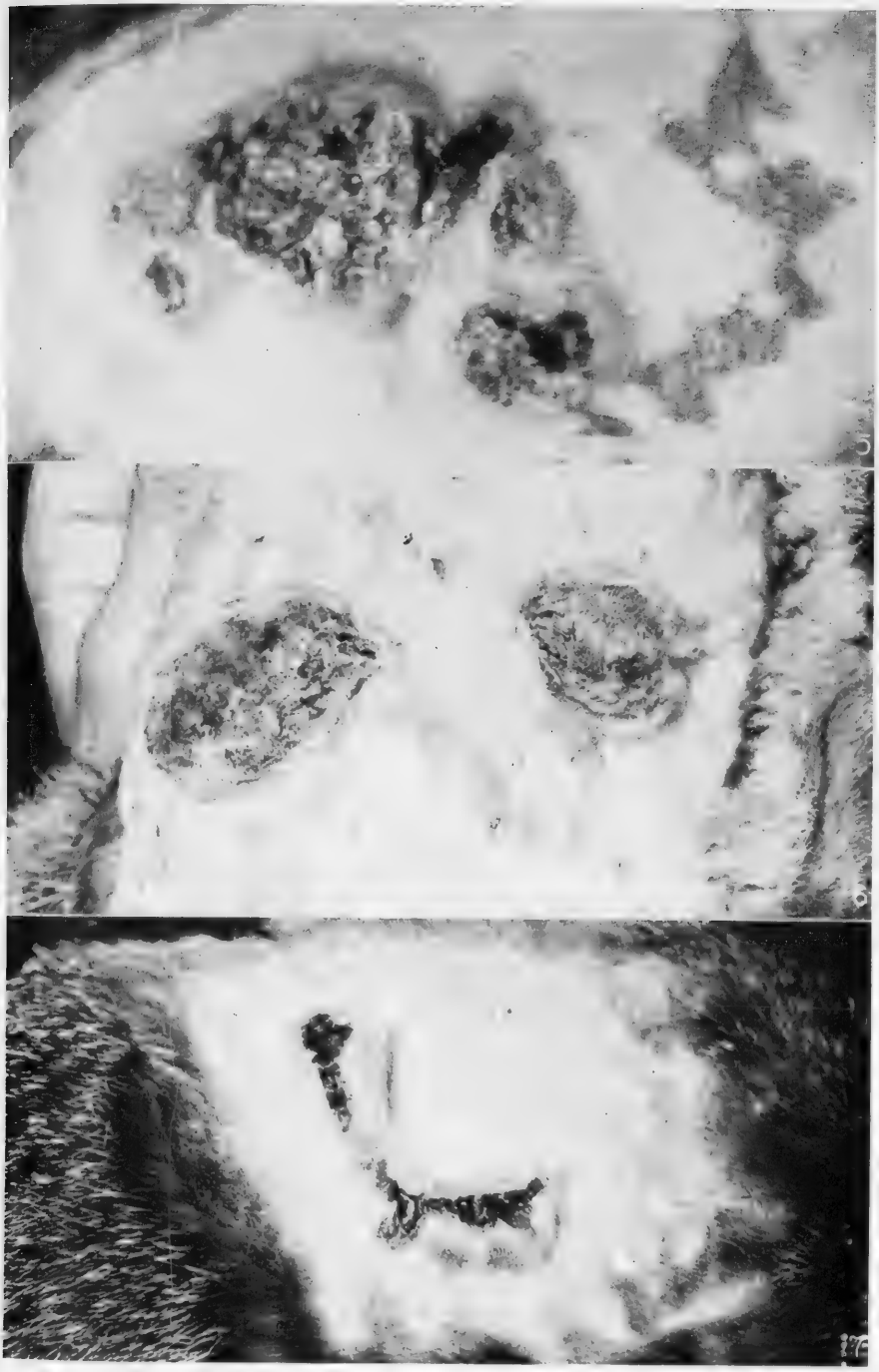
FIG. 5. Confluent syphilitic papules produced on skin the seat of coal tar dermatitis. Taken 63 days after inoculation.

FIG. 6. Two chancres produced by inoculating a single granulating wound.

FIG. 7. Result of attempt to produce an L-shaped chancre by inoculating an L-shaped granulating wound.



(Chesney and Kemp: Experimental syphilis. II.)



(Chesney and Kemp: Experimental syphilis. II.)

STUDIES ON UROBILIN PHYSIOLOGY AND PATHOLOGY.

I. The Quantitative Determination of Urobilin.

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Numerous methods for the quantitation of urobilin have been reported and a great many estimations have been made with their aid in clinical conditions. There has been no semblance of accord among workers as concerns the origin and fate of the pigment; and analysis of the literature[1,2] accounts for this in making clear the conflicting nature of the evidence accumulated. On the whole it is not surprising that interest in the pigment has lagged in recent years despite a general feeling among workers that it has a weighty significance.

The possession of an experimental method[3] adapted to a precise study of bile, an element supposedly of primary importance in the metabolism of urobilin, has led us to take up the study of it. The present paper deals with the quantitative estimation of the pigment in bile, urine, and stool. Subsequent papers will be concerned with its variations under experimental conditions. The term urobilin as used in these studies will be taken to include all of the reduction products of bilirubin capable of causing a green fluorescence in alcoholic zinc acetate solution. With urobilinogen, as such, we have not been concerned, since it is easily oxidized to the more readily recognizable urobilin.

The discovery in 1869 of urobilin was made possible by its spectroscopic properties. Jaffe[4] while examining a specimen of urine noted an absorption band

[1] Meyer-Betz, F., *Ergebn. inn. Med. u. Kinderheilk.*, 1913, xii, 733.

[2] Eppinger, H., Die Hepato-lienalen Erkrankungen, Berlin, 1920, 76.

[3] McMaster, P. D., Broun, G. O., and Rous, P., *J. Exp. Med.*, 1923, xxxvii, 395.

[4] Jaffe, M., *Virchows Arch. path. Anat.*, 1869, xlvii, 405.

lying between Fraunhofer's lines *b* and *F*. He later found it to be more marked in urines from patients suffering from certain febrile diseases. In normal human bile the same phenomenon was observed. From its occurrence in both the urine and bile Jaffe gave the name urobilin to the pigment responsible for the band. He described its presence in normal stools as well,[5] confirming the original observation by Vanlair and Masius.[6]

The interest which the new substance aroused was very great. Investigations of it have been extensive and many workers have described its properties and its relation to the other bile and blood pigments. Without going into detail on the subject, one may summarize what we know of the chemical nature of urobilin in saying that it is a reddish brown pigment derived from bilirubin by reduction in the body, or from urobilinogen by simple oxidation *in vitro*. Urobilinogen, the usual reduction product of bilirubin, is a normal constituent of feces and bile, and, sometimes, in traces, of the normal urine. As the chromogen of urobilin it is spontaneously oxidized to it in the air; and it is identical with the hemopyrrols of the stool.[1,7]

Urobilin is intensely fluorescent in alcoholic zinc acetate solution,[8] characteristically showing a bright green color by reflected light, though in transmitted light it is brown or pink, depending on the concentration. It has characteristic absorption bands, is sensitive to oxidizing agents, and to acids and alkalies, and, in the animal body, probably exists in varying stages of oxidation and polymerization.

Previous Methods of Urobilin Determination.

Previous methods have been reviewed adequately by several authors.[1,9,10] Mention must be made of two, however, which have been largely used in the past. One of these depends on the spectroscopic absorption bands, the other on the development of its fluorescence. By both methods the urobilin-containing solution is diluted until the characteristic manifestation is no longer visible; and the estimation is reported in dilution values. At the outset of our studies both methods were tried and it soon became apparent that the end-point was in both cases highly indefinite. This was especially true of fluorescence, which is scarcely surprising since the threshold of visibility is notoriously so variable. General fatigue,

[5] Jaffe, M., *Centr. med. Wissensch.*, 1871, ix, 465.
[6] Vanlair and Masius, *Centr. med. Wissensch.*, 1871, ix, 369.
[7] Fischer, H., and Röse, H., *Z. physiol. Chem.*, 1912, lxxxii, 391.
[8] Schlesinger, W., *Deutsch. med. Woch.*, 1903, xxix, 561.
[9] Wilbur, R. L., and Addis, T., *Arch. Int. Med.*, 1914, xiii, 235.
[10] Adler, A., *Deutsch. Arch. klin. Med.*, 1922, cxxxviii, 309.

fatigue of the eye, subjective elements, psychic influences all play a part in the results of the determination. We were obliged, therefore, in the beginning, to discard methods based on the threshold of visibility despite the fact that most of the work on record has been performed with their aid. There is one really accurate method of measuring intensity of the fluorescence or of the absorption bands of urobilin by use of the spectrophotometer.[11] But the instrument is elaborate and seemed unadapted to our plans.

Recently, Pincussen[12] has compared fluorescent solutions of urobilin with a standard made by dissolving fluorescein in water. He prepares a series of dilutions of the latter substance, and by comparing them with his unknown specimen arrives at its value in terms of the strength of fluorescein. He mentions the reduction of this value to terms of mg. of urobilin but presents no detailed data thereon.

One can scarcely assume that a solution, because its color is twice as strong as that of another, contains twice the amount of pigment. Even granting a definite relation between intensity of fluorescence and weight of pigment, one cannot say that such curves of fluorescein and urobilin run parallel. Pincussen, furthermore makes no mention of controls on the reaction and on the concentrations of zinc acetate and alcohol, factors which have been shown[13] to influence markedly the intensity of the color.

Present Method.

In the study of urobilin as derived from the body one is confronted with two problems. The first concerns the extraction and separation from interfering pigments, the second the method of quantitation itself. The difficulties of this latter have been indicated in a general way above; they have been overcome, we believe, in the method now to be described.

Our method is, like that of Pincussen,[12] one of comparative fluorescence, but the unknown factor of the relationship of the intensity of fluorescence to content of urobilin has been eliminated. A certain amount of acriflavine is dissolved in water and the zinc acetate filtrate from the urobilin-containing solution is diluted till it matches this color. The standard itself is calibrated against the fluorescence

[11] Charnas, D., *Biochem. Z.*, 1909, xx, 401.

[12] Pincussen, L., *Deutsch. med. Woch.*, 1922, xlviii, 1074.

[13] Marcussen, S., and Hansen, S., *J. Biol. Chem.*, 1918, xxxvi, 381.

of a standard solution of urobilin. The method of obtaining the clear zinc acetate filtrate and acriflavine standard will be described further on.

The fluorescence of urobilin is notably affected by the reaction and the concentrations of the alcohol and zinc acetate employed to develop it. A somewhat detailed investigation in the matter has been made recently by Marcussen and Hansen,[13] who conclude that any concentration of zinc acetate over 2.5 per cent and of alcohol above 50 per cent will bring out the maximum fluorescence, while the point of greatest color is to be found at an acidity equivalent to that of 0.05 N HCl. Our reagents have been so made as to yield approximately these concentrations and this acidity in the final filtrate. Furthermore a standard diluting solution has been used throughout the determinations.

The standard diluting solution consists of 60 per cent alcohol 2,000 cc., zinc acetate 50 gm., and concentrated hydrochloric acid 2 cc., filtered repeatedly till perfectly clear. 15 cc. portions are measured into test-tubes of uniform length and bore, and graduated amounts of the fluorescent solution of urobilin added, say 0.50, 0.55, and 0.60 cc., and the tubes gently shaken to mix the contents. These solutions are compared in color with the standard acriflavine solution placed in test-tubes of the same type and arranged in a row alternately with the unknowns at intervals of 3 cm., in a dark box placed in a room with subdued light. Illumination to show the fluorescence comes from a 200 watt daylight lamp situated 1 meter above the row of tubes, which are examined for their color through a a horizontal opening in one wall of the box opposite the end along which the tubes are arranged in a slotted block fitted to receive them. The axis of vision is thus at right angles to the line of illumination, which makes manifest the fluorescence most advantageously.

The type of box adopted is similar to that described by Adler.[10] The inner walls were painted a dull black and measured 21 by 28 by 16 cm. The eyepiece from an ordinary stereoscope is fitted to the opening, through which the examination of the solutions is made. The slotted block fitted along the other end of the box has six grooves into which 14 mm. tubes are fitted snugly at intervals of 3 cm. A removable cover is provided which contains six round holes bored just over the slots in the block below. Through them the tubes could be inserted or removed at will with the cover intact. They were about 16 cm. long and projected outside 2 to 3 cm. above the box.

The point at which the color match comes closest is noted and the dilution value of the solution under test is calculated. If the unknown, when diluted as indicated above, is still too weakly colored for correspondence with the standard, more of the filtrate is added in 0.10 cc. increases till one of the tubes matches properly. If, on the other hand, the first dilution yields too intense a green, it is advisable to make a preliminary dilution of the zinc acetate filtrate of say 1 to 4, depending upon how deeply fluorescent it is. The dilution factor is, of course, multiplied

into the final value. In this way the error of small additions is avoided; and amounts under 0.30 cc. were, therefore, never used.

Calculation of the Dilution Value.

The dilution value of the fluorescent zinc acetate filtrate must always be multiplied by a factor dependent upon the number of dilutions involved in developing its color. Thus with bile, 8 is the characteristic factor since 20 cc. of the secretion was used, diluted to 80 cc. during clearing and the volume doubled in the final zinc acetate filtrate. With urine it is 2 since each specimen is diluted but twice before its pigment content is measured. When it has first to be cleared as described below, the factor is 5 instead of 2. Similarly with stool specimens, 8 always enters into the final figure. In each case, of course, the value obtained is multiplied according to the amount of the daily specimen.

Standard Fluorescent Solution.

Among a number of fluorescing substances tried, acriflavine—3.6. diamino acridine methochloride,[14] Boots Pure Drug Company—was found best suited to the work. Its solution by direct light has a color not unlike that of urobilin, and its fluorescence on dilution is identical in appearance with that of the pigment. In order to avoid a possible interference by contaminants as well as to increase the delicacy of the reading an end-point of great dilution was adopted. A solution containing 1 mg. in 30,000 cc. of water proved strong enough to give a color that could be used as a standard, giving as it did a well defined green color when viewed in the dark box. Such a solution was found fairly stable under ordinary circumstances if kept away from the light. The parent solution of a 1/1,000 strength was kept in a cool dark place and new dilutions were made up every week, 15 cc. portions of which were placed in the standard tubes at each reading. These solutions were always made up with distilled water which was filtered before using.

Separation of Urobilin and Development of Its Fluorescence.

Before quantitating urobilin according to the method above outlined, it is necessary to recover this pigment from the body fluids containing it and to have it dissolved in alcoholic zinc acetate, for only in such a solution is the fluorescence displayed. The procedures finally adopted to this end are as follows:

Urine.—A portion (25 cc.) of the dog's 24 hour specimen was taken, made neutral or barely acid to litmus, and saturated zinc acetate in 95 per cent alcohol was added up to 50 cc., after throwing in about a gm. of the dry powder to insure an excess of the salt. The mixture is shaken and then filtered till clear. A drop or

[14] Benda, L., *Ber. chem. Ges.*, 1912, xlv, 1787.

two of tincture of iodine is added to oxidize all the urobilinogen to urobilin, which is then manifest through the green fluorescence visible by reflected light.

In some instances, as when bilirubinuria is intense, a preliminary clearing may be necessary. The specimen can be cleared the same way as bile by a method to be described further on, but it was found simpler to use the supernatant fluid obtained after a precipitation such as is employed to obtain bilirubin for quantitation.[15] For this purpose calcium bilirubinate is thrown down with calcium chloride in alkaline solution and recovered by centrifugation. The relatively clear supernatant liquid contains practically all of the urobilinogen, as was later found by a comparison with other methods of clearing. After neutralization a 25 cc. portion is treated like an ordinary specimen, as described above. Since this procedure involves a dilution of the original urine sample from 20 to 50 cc. the factor 2½ figures in the final estimation of the dilution value.

Stool.—Urobilin is here present as adsorbed matter and the problem is to divide the stool so finely as to enable the extracting solution to withdraw the pigment completely. Adler,[16] investigating this point at some length, found that on simple trituration in a mortar with acid alcohol so large and constant a percentage of the urobilin comes out with the first extraction that for general purposes repeating the process is quite unnecessary. We have preferred to agitate the total stool specimen immediately after its collection with water in an ordinary milk shaker machine. This, within 2 or 3 minutes, breaks up the fecal masses and yields a finely divided emulsion. Each specimen so obtained is made up to 250, 500, 750, or 1,000 cc. with water, depending on its bulk and supposed content of pigment. Aliquot (25 cc.) portions thereof are shaken in a mechanical shaker for an hour with 75 cc. of acid alcohol (1,600 cc. 95 per cent ethyl alcohol, 25 cc. concentrated HCl mixed and made up with distilled water to 2,500 cc.) They are then left overnight at room temperature to allow oxidation of the chromogen to urobilin as suggested by Wilbur and Addis.[9] Since the material is now in alcoholic solution further bacterial action is precluded.

The next day the mixture is shaken and 25 cc. portions are taken, about a gm. of dry zinc acetate is added to each as an excess, and they are made up to 50 cc. with saturated zinc salt in 95 per cent alcohol. Shaking is now done, and the fluid repeatedly filtered till clear. After adding a drop or two of tincture of iodine it is examined for its fluorescence.

Bile.—The reported methods of clearing bile by ridding it of bilirubin pigment are numerous, but trial of them has in the past given imperfect results. For the agent if efficient in absorbing the bilirubin as is Fuller's earth[9] or charcoal, holds back urobilin likewise, while if urobilin comes through, as after treatment with calcium hydrate,[17] or talc,[18] the filtrate is deeply yellow with bilirubin.

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[15] Hooper, C. W., and Whipple, G. H., *Am. J. Physiol.*, 1916, xl, 332.

[16] Adler, A., and Schubert, E., *Biochem. Z.*, 1922–23, cxxxiv, 533.

[17] Brand, J., *Arch. ges. Physiol.*, 1902, xc, 491.

[18] Auché, A., *Compt. rend. Soc. biol.*, 1908, xlv, 758.

Advantage has been taken, in the method finally adopted, of the extreme solubility of urobilin in ammonia and the adsorbability of flocculent ferric hydroxide for biliverdin as pointed out by Adler.[10] If ferric chloride be added to normal dog bile the color changes from brown to green because of the oxidation of bilirubin to biliverdin. The urobilinogen must simultaneously be changed to urobilin. If now the ferric hydroxide is thrown down as a flocculent precipitate with an excess of ammonia water and filtration immediately carried out a perfectly clear solution comes through, tinged brown or reddish from its content of urobilin, which remains in solution only if the filtrate is ammoniacal. In urobilin-free biles, as will later be shown, the filtrate is colorless.

As routine 20 cc. of 20 per cent ferric chloride is mixed with 20 cc. of fresh dog bile, in a beaker, 40 cc. of 20 per cent ammonia water immediately added, and the mixture stirred and filtered at once into a 50 cc. graduate. When 25 cc. of fluid has been obtained the remainder is discarded. The order in which the reagents are added is important: if the ammonia be added before the ferric chloride the filtrate will not be clear.

To 25 cc. of filtrate concentrated HCl is added drop by drop till the solution is barely acid to litmus, about a gm. of dry zinc acetate is thrown in, and the whole is made up to the 50 cc. mark with saturated zinc salt in 95 per cent alcohol. The filtrate from this mixture will show the green fluorescence of the urobilin contained in the original bile sample.

Expression of Findings in Terms of Mg.

To express the findings in mg. it was necessary to determine the dilution value of a known amount of pure urobilin examined under the ordinary conditions in the standard dilutant. After repeated attempts a specimen of high purity was obtained as follows:

Heavily pigmented dog stools were selected and extracted with ether to remove most of the fatty acids, and lipoids, indole and scatole. The residue was then ground up and whirled in a motor-driven milk shaker with acid alcohol, agitated in a mechanical shaker for an hour, and centrifuged. The clear supernatant brown liquid was poured off and one-third its volume of chloroform added. This gave a clear mixture. Shaking in a separatory funnel with water brought down the lower chloroform layer and this was washed once or twice with water, passed through a filter paper in a funnel containing anhydrous sodium sulfate to remove the water, and evaporated over a warm water bath, in an Erlenmeyer flask. The dark sticky residue was taken up in 20 per cent ammonia water with which it formed a clear solution, and ice was added. Precipitation of the urobilin was then effected by the slow addition of concentrated sulfuric acid while the solution was kept cold by further additions of ice. The formation of ammonium sulfate is known to throw down the pigment completely as the solution becomes acid. The

flocculent precipitate thus formed was filtered off, washed several times with water, and allowed to dry.

This residue was obviously impure, still having a faint fecal odor. It was washed with ether which extracted lipoids as well as a good deal of pigment other than urobilin; and, after drying again, it was extracted with chloroform, filtered, and allowed to evaporate on a large watch crystal over a warm water bath. The dry film of urobilin scraped off had the form of thin glistening, non-crystalline plates. It was perfectly hard and dry, and after grinding in a small agate mortar yielded a homogeneous, impalpable, odorless powder of dark brown color. It was soluble in alcohol and chloroform, insoluble in ether, wood alcohol, and benzene. It could not be dissolved by water but if a few drops of ammonia water were added it went immediately into solution.

The urobilin thus prepared was assumed to be pure, and, however this may have been, it was sufficiently so to serve as a standard in the work for we have been concerned only with relative changes in urobilin values. The absolute determination of this pigment has been, for us, of little significance. The purified substance was intensely fluorescent, a tiny particle in alcoholic zinc acetate giving rise to a most intense green color. In dilution values 1 mg. in 950 cc. of the standard dilutant matched the fluorescence of the acriflavine standard, which contained 1 mg. of this substance in 30,000 cc. of distilled water, the end-point selected for the final comparison throughout all the determinations.

It was easy now simply to divide all dilution values by 950 and obtain an equivalent in approximate if not actual mg. of urobilin. Thus the possibility of error due to changes in the pH, alcoholic or zinc acetate concentrations was eliminated. The final estimation of color in every case, including that of the pure urobilin pigment itself, was performed in the same solution and under the same conditions. The expression of the values in terms of mg., therefore, involves no correction due to these factors.

Duplicate and repeated readings of the same urobilin specimen were carried out and agreement within 10 per cent was found to be the rule. When a zinc acetate filtrate matched the standard directly, that is to say, without any dilution there can have been present in it only 1 mg. of urobilin for every 950 cc. or, if one take a concrete case, in a bile sample of 100 cc. less than 1 mg. Specimens containing amounts under this could not be quantitated, but traces were roughly estimated by the use of a concentrated beam of light. The routine use of the strong light beam gave proof of the presence or absence of the pigment. Urobilin-free solutions were those which showed no color even with intense illumination.

DISCUSSION.

The difficulties of urobilin quantitation are considerable, as has already been sufficiently pointed out. There has been no simple, clinically useful method free from objections. It is very largely this absence of a satisfactory means for measurement which has retarded precise investigations of the pigment. In the method outlined above a degree of accuracy has been attained, it is believed, which enables one to estimate the amount of pigment satisfactorily; though exactitude cannot be expected until more is known of its chemical nature. Fortunately exactitude is not necessary for studying the physiology and pathology of urobilin. It is sufficient to be able to determine whether the substance is present, and to follow with a fair degree of accuracy its relative variations. These requirements have been met in the methods devised by us. Filtrates matching our standard solution contain only 0.009 mg. of urobilin per cc. The substance is fluorescent under a concentrated beam of light in far greater dilution than this, so great indeed that when the phenomenon cannot be observed one may safely conclude that to all intents and purposes urobilin is absent.

Some of the urobilin must be lost in the extraction and clearing methods. In the case of the stool not more than 10 to 20 per cent is left behind after a mere trituration;[10] and far less can have escaped our method of treatment. Little is lost in the urine because it is usually examined directly. But with bile the loss may be considerable. A comparison of various methods of clearing leads us to believe that over 75 per cent of the urobilin is recovered from specimens cleared with ferric hydroxide. As already mentioned, duplicate determinations were made on a large series of specimens and agreement within 10 per cent was always the result. 1 or 2 days delay in reading, made no difference when the specimen had been kept in a stoppered vessel in a dark cool place.

The value of fluorescence as an ultimate measure of urobilin depends on the manner in which it is utilized. For the difficulties encountered when this is not properly done one may consult the recent discussion between Adler[19] and Pincussen.[20] In the method here presented

[19] Adler, A., *Deutsch. med. Woch.*, 1922, xlviii, 1442.

[20] Pincussen, L., *Deutsch. med. Woch.*, 1922, xlviii, 1443.

only one assumption has been made; that two urobilin solutions examined under the conditions we have described and showing the same degree of fluorescence, on comparison with an acriflavine standard, contain the same amount of pigment.

In the ultimate quantitation of the material it would have been best, of course, to use weighed amounts of urobilin itself as the standard. This substance is, however, too unstable for use over long periods of time, and moreover its preparation is difficult and tedious. Acriflavine is a convenient and satisfactory substitute. The fluorescence is stable and practically identical in hue with that of urobilin. No difficulty at all was experienced in making a color match.

The method has proved easy to carry out and ideally suited for routine laboratory estimations on material from the dog. Whether it will prove equally suited to clinical purposes remains to be determined.

SUMMARY.

Methods are presented for the development of clear fluorescent solutions from urobilin-containing urine, feces, and bile obtained from the dog. Conversion of the urobilinogen to urobilin is accomplished during the procedures. Measurement of the urobilin content is effected by comparing its fluorescence at great dilution with a standard containing acriflavine, calibrated in turn against pure urobilin.

Means have been found to avoid the influence of the factors responsible in part for the inaccuracy of urobilin determinations. The method will quantitate exceedingly small amounts of urobilin and estimations on the same material agree within 10 per cent.

Though designed for experimental procedures on the dog, the methods would seem adapted for clinical uses as well.

STUDIES ON UROBILIN PHYSIOLOGY AND PATHOLOGY.

II. Derivation of Urobilin.

Relation of the Bile to the Presence of Urobilin in the Body.

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In this paper, and in others to follow, we propose to set forth facts regarding urobilin which have become evident through the use of a method peculiarly favorable to precise conclusions. The present work will be concerned with the problem of the normal physiology of the substance. In later communications the influence of pathological conditions will be taken up. Since the discovery of urobilin[1] clinical interest in the substance has always been great. Yet at the present day the conclusions confidently drawn by workers with the substance far outrun actual knowledge of it. Few extensive experimental studies have been made and these are open to well grounded criticism.

By permanent intubation of the bile ducts of animals[2] the total liver bile can be obtained in a sterile state during a period of months. This method has already been utilized for the study of bile constituents[3–8] and it would seem especially suited for experiments on the vexed question of the relation of the secretion to urobilin. Variations of the method can be successfully put into application. Thus, for example, one may drain to the outside the bile from only one lobe of the liver, or may so arrange the intubation as to be able, at will, to collect the total bile, or have it flow as usual to the intestine.

[1] Jaffe, M., *Virchows Arch. path. Anat. u. Physiol.*, 1869, xlvii, 405.
[2] Rous, P., and McMaster, P. D., *J. Exp. Med.*, 1923, xxxvii, 11.
[3] McMaster, P. D., Broun, G. O., and Rous, P., *J. Exp. Med.*, 1923, xxxvii, 395.
[4] Rous, P., Broun, G. O., and McMaster, P. D., *J. Exp. Med.*, 1923, xxxvii, 421.
[5] McMaster, P. D., Broun, G. O., and Rous, P., *J. Exp. Med.*, 1923, xxxvii, 685.
[6] Broun, G. O., McMaster, P. D., and Rous, P., *J. Exp. Med.*, 1923, xxxvii, 699.
[7] Broun, G. O., McMaster, P. D., and Rous, P., *J. Exp. Med.*, 1923, xxxvii, 733.
[8] McMaster, P. D., *J. Exp. Med.*, 1924, xl, 25.

PREVIOUS WORK.

The previous literature is crowded with articles written in advocacy of one hypothesis or another of urobilin formation. There are enterogenous, hepatogenous, histogenous, hematogenous, and even nephrogenous hypotheses of its genesis. The conflicting evidence has been admirably sifted and weighed by Meyer-Betz.[9]

As far back as 1871 Maly[10] succeeded in obtaining urobilin from bilirubin by reduction *in vitro*. Since the work of Friedrich Müller[11] it has been known that a bacterial reduction of bile pigment in the intestine accounts for part at least, of the urobilin of the stool. Recently Kämmerer and Miller[12] have demonstrated that this change can be effected *in vitro* by organisms of the type of *B. putrificus* with the aid of *B. coli* together with other aerobes in the presence of protein decomposition. It has been noted by many workers in the past that the feces of patients, or dogs, operated upon for the formation of bile fistulas and, hence, losing all the bile, became entirely, or almost urobilin-free in a short time. However, there was usually a reappearance of the pigment in smaller amounts a few days later. In spite of this discrepancy, the authors already mentioned, as well as Fischler,[13] Wilbur and Addis,[14] Adler,[15] and many other workers in this field are agreed that the urobilin of the stool is largely derived from the bilirubin of the bile, and has an enterogenous origin in that it is formed out of bilirubin in the intestine.

The hypotheses concerning the derivation of the urobilin which is present in the bile itself, show no semblance of accord. The enterogenous derivation of the urobilin in the stool, has suggested to many that this pigment, when present in the bile, takes its origin from the same source. According to their belief urobilin formed by reduction of the bilirubin in the intestine, is in part absorbed and resecreted by the liver into the bile. The data for and against this view have been well summarized by Meyer-Betz.[9]

The existence of urobilin in the bile has convinced some observers that the liver possesses the ability to manufacture the pigment, especially under the conditions of disease. Fischler[13] studying dogs with ordinary bile fistulas believed he had been confronted with an occasional true hepatogenous formation of the pigment. The possibility of an hepatic derivation of the urobilin in the bile has recently been accepted by Whipple on the basis of his own work.[16,17]

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[9] Meyer-Betz, F., *Ergebn. inn. Med. u. Kinderheilk.*, 1913, xii, 733.

[10] Maly, R., *Ann. Chem.*, 1872, clxiii, 77.

[11] Müller, F., *Verhandl. Schles. Ges. vaterl. Cult.*, January 15, 1892.

[12] Kämmerer, H., and Miller, K., *Deutsch. Arch. klin. Med.*, 1923, cxli, 318.

[13] Fischler, F., Physiologie und Pathologie der Leber, Berlin, 1916.

[14] Wilbur, R. L., and Addis, T., *Arch. Int. Med.*, 1914, xiii, 235.

[15] Adler, A., *Deutsch. Arch. klin. Med.*, 1922, cxxxviii, 309.

[16] Whipple, G. H., *Physiol. Rev.*, 1922, ii, 440.

[17] Whipple, G. H., *The Harvey Lectures*, 1921–22, xvii, 95.

In studies on the physiology of urobilin one great source of error has been constantly present, the faulty bile collection inevitable to work with the open fistula. The method has in general necessitated part time collections, which are by no means trustworthy as a gauge of the 24 hour output. Often the animals have been allowed to lick their fistulæ with the result that bile has reached the intestine in greater or smaller amounts thus accounting, as will be shown further on, for the reappearance of urobilin in the bile and stool. The secretion is obtained under conditions that favor infection, gall bladder inflammation, cholangitis, and hepatitis. Pigment changes in the bile due to action of the bacteria upon it cannot be ruled out, and some workers have held them responsible for the findings. The gall bladder is capable of modifying the bile greatly[18] and the secretion studied has always been submitted to its influence. By the method we have employed all these difficulties are avoided.

EXPERIMENTAL FINDINGS.

It has long been known that under normal conditions in the dog[19] and man[1] urobilin is present in the stool and bile. We have regularly found it in the case of the normal dog. Our first problem has been to determine the normal variations of fecal and urinary urobilin and then to study in the same individuals the effect of bile deprivation.

For the preliminary observations healthy, vigorous dogs were employed. They were kept, throughout the period of study, in metabolism cages but were daily allowed a brief run in a small yard with a clean tile floor. The diet given consisted, in the main, of the standard bread and milk and lean meat used in previous experiments,[3-8] that is to say, 1,050 gm. of bread wet with milk and 450 gm. of lean meat, for an animal of about 12 kilos. However, in many instances a free diet of table scraps and bones was given. This difference in ration appeared to be without effect on the urobilin output of the animals.

It was found that dogs which were let run for a few minutes every day soon acquired the habit of defecating then and then only, with result that the cage urine was not contaminated with feces. This proved of great advantage in the work. The findings when contamination occurred, as occasionally happened, were ruled out. The tiled floor of the small exercise yard was cleaned prior to

[18] Rous, P., and McMaster, P. D., *J. Exp. Med.*, 1921, xxxiv, 47.

[19] Beck, A., *Wien. klin. Woch.*, 1895, viii, 617.

the loosing of each dog. Such small amounts of urine as were voided in it were taken up into a pipette and added to the 24 hour specimen by an assistant, who observed each animal closely during the exercise period. The incidental loss of urine occasionally introduced a slight error into the urobilinuria readings but the complete separation of stool and urine removed a far greater one. In working with dogs intubated for total bile collection, the stool is urobilin-free as will be shown further on and fecal contamination of urine specimens does not present a difficulty.

Quantitative daily estimations of the urobilin in urine and feces were made as routine. The methods have already been described.[20] It may be repeated here that separate determinations of urobilinogen and urobilin were not attempted, but the former was oxidized to the latter during the process preliminary to quantitation. Bilirubin was quantitated, in bile and urine, by a modification[3] of the method of Hooper and Whipple.[21] The hemoglobin percentage of the peripheral blood was followed by both Newcomer's method[22] and that of Palmer.[23]

Normal Urobilin Variations.

Great care must be taken in the interpretation of variations in the urobilin output by way of the stools. Changes in intestinal motility are responsible for startling differences in the readings. Both Wilbur and Addis[14] and Dean[24] have remarked upon the effects of constipation and diarrhea on the quantity of urobilin in human stools. Some of our experimental animals were given morphine sulfate for its constipating effect. The decrease in the amounts of urobilin of the stools passed after several days constipation, as thus brought about, was striking. The same phenomenon was many times noted incidentally to constipation occurring in the course of other experiments. The daily urobilin output even after defecation was recommenced, showed a very great decrease during 3 to 4 days, from that yielded in the period preceding the constipation. No protocols of such cases need be given here as there seems to be a general agreement concerning them. Whether the decreased elimination is significant of absorption of the pigment from the bowel, or its destruction therein, is not clear. Certain it is that the phenomenon acts to lessen greatly

[20] Elman, R., and McMaster, P. D., *J. Exp. Med.*, 1925, xli, 503.
[21] Hooper, C. W., and Whipple, G. H., *Am. J. Physiol.*, 1916, xl, 332.
[22] Newcomer, H. S., *J. Biol. Chem.*, 1919, xxxvii, 465.
[23] Palmer, W. W., *J. Biol. Chem.*, 1918, xxxiii, 119.
[24] Dean, R. S., unpublished data.

the value of fecal urobilin estimations. When the passage through the intestines is more rapid than usual, greater amounts of urobilin are to be found in the feces, but again whether this is due to a lack of absorption or a lessened destruction is not clear.

The Effects of Total Bile Loss on Urobilin.

After the day-to-day variations in the output of urobilin in these normal dogs had been determined, we took steps to find what happens when the the bile is diverted from the intestine and lost to the body. Some of the animals already studied were operated upon and the common duct was intubated by the method of Rous and McMaster,[2] by which means all of the bile can be obtained in a sterile condition during months. The animals remained in excellent condition.

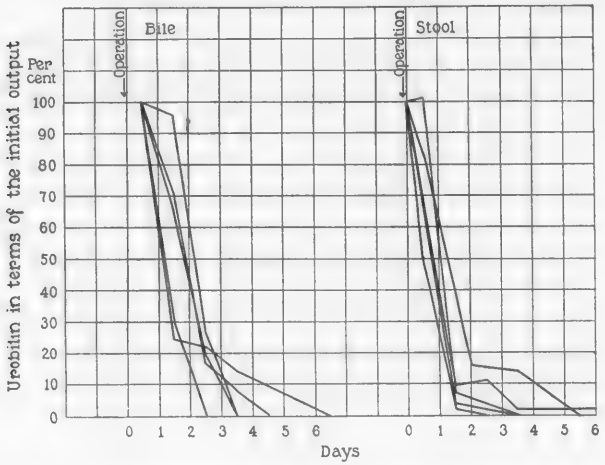
Operations were conducted under ether anesthesia. For some time previously the output of urobilin in feces and urine had been followed, and the hemoglobin percentage of the blood had been ascertained daily. After operation 24 hour specimens of the bile were collected from the balloon each morning and this bile, the urine, and feces were daily analyzed for bilirubin as well as for urobilin and the observations on the hemoglobin were kept up. Cultures were daily made of the bile, and centrifugalized sediments from it were frequently stained and examined for bacteria. All cases in which the hepatic bile became infected were ruled out.

At the end of the experimental period the animals were killed with chloroform and careful autopsies made with special reference to reconstitution of ligated ducts or anastomoses which might have developed around the cannulas. Both injection methods and dissection were utilized for the purpose. The few instances showing these causes of possible error were ruled from consideration, as were the very occasional ones showing pathological change in the liver or other organs.

Fourteen dogs with intubated common duct were observed. In twelve of these in which the bile remained sterile and total bile deprivation was effected, so that no pigment reached the intestine, the urobilin rapidly disappeared from bile and stool, and none was again found even though the observations were continued as long as $4\frac{1}{2}$ months.

The day after the intubation a large amount of urobilin was regularly found in the bile, but it decreased rapidly, afterwards, and within 2 to 4 days all traces of it had disappeared. In the stools the pig-

ment disappeared almost as rapidly, the specimens being completely acholic and urobilin-free within 3 days after operation. Text-fig. 1 represents the findings in several animals chosen at random. In this chart the average of the urobilin findings in the feces of the days before operation, is plotted as 100 per cent, and on the days after operation, in terms of this figure. The output of urobilin of the bile on the 1st day after operation is plotted as 100 per cent and the sub-



TEXT-FIG. 1. The disappearance of urobilin from bile and feces of animals after intubation of the common duct.

Text-fig. 1. This illustrates the rapid decrease in the urobilin of the bile and stool after the operation for intubation of the common duct for the collection of the total bile. No bile pigment was allowed to reach the intestine. The bile and feces remained urobilin-free during the remainder of the period of observation. Each line represents the findings in an animal.

sequent changes in terms of this. The normal dogs showed no urobilinuria prior to operation, and after it the pigment was present in the urine for 1 or 2 days only and then was not found again.

In three of the fourteen animals urobilin persisted in the stools, always though in amounts too small to determine quantitatively, at the most in very faint traces. The bile of such animals was altogether urobilin-free. Realizing the possibility of the presence of

minute bile ducts which had escaped ligature and severance, or of the development of small anastomotic branches, very careful dissections and injections were made at autopsy but no such channels were found. In the intestinal mucosa, however, were yellowish areas which yielded the reaction for bile pigments with fuming nitric acid. Similar findings of traces of urobilin in the stools have been reported by other workers with bile fistula dogs and have been considered by some as a typical finding.

Gerhardt[25] stated that the cells of the intestinal mucosa, of bile fistula dogs having traces of urobilin in the stool, showed globules containing bilirubin which was apparently being secreted into the intestines. Fischler[13] who cites Gerhardt substantiates this finding. As will be seen later in the present work, these traces of urobilin become far more noticeable in dogs with total biliary obstruction and a resulting outspoken tissue icterus. In such animals the intestinal mucosa is frankly yellow, a passage of bilirubin into the intestine can readily occur and urobilin is demonstrable in the stool. Whatever the significance of faint traces of urobilin in the stools in our fistula dogs, they were found in so few and the amount of urobilin was so slight that it could scarcely play an important part in the metabolism of the substance. Experiments to be reported below will show that traces of blood in the intestine cannot be held accountable in any way for this urobilin.

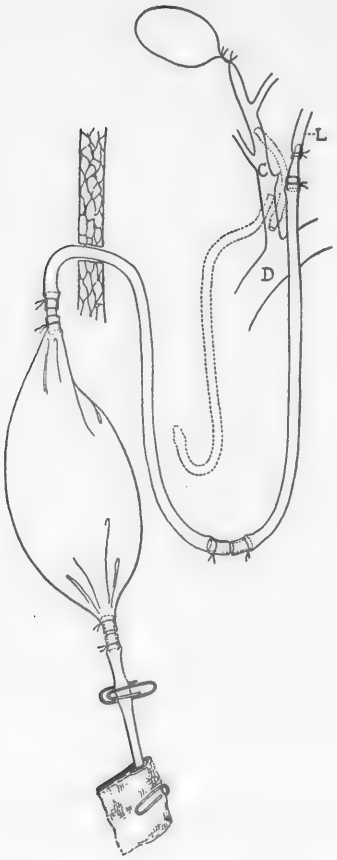
Needless to say, loss of the bile introduces an abnormal state of affairs. In the further study of this pigment we made attempts to approach the natural conditions closely.

The Effects of Partial Bile Loss.

To obtain samples of the bile while allowing most of it to flow, as normally, to the intestine a modified form of intubation was employed, a sampling intubation one may call it, whereby the secretion from only one liver lobe, or at most from two small ones, was poured into the balloon. In this way not more than 25 or 30 per cent of the bile was lost to the animal, the remainder passing through the normal channels into the intestine.

In nine cases the intubation was effected by joining one of the branches of the hepatic duct to the collecting apparatus, as shown in the diagram, Text-fig. 2.

[25] Gerhardt, D., *Z. klin. Med.*, 1897, xxxii, 303.



TEXT-FIG. 2. Diagram of the schemes of intubation of bile ducts for the collection of part of the bile.

Text-fig. 2. The diagram shows in continuous lines the arrangement for intubation of a single liver lobe usually used. When the individual ducts were small another scheme was occasionally employed (dotted lines). The common duct was ligated above the entrance of the duct from the lateral liver mass (right lateral and caudate lobes) and a cannula was inserted so as to drain bile from this mass only. A glass "bridge" cannula placed in the common duct conducted the bile from the main liver mass around the obstruction and passed it on toward the intestine. *L*, left lateral bile duct; *C*, common duct; *D*, duodenum.

It was necessary to use a cannula of only slightly smaller bore than ordinary. In two animals bile from the right lateral and caudate lobes was collected by intubating the common duct just below the entrance of the small duct from these, and then "bridging" the common duct with a glass tube in such a way that the bile from the main mass of the liver flowed around the obstruction made by the intubation, back into the common duct, and so into the intestine.

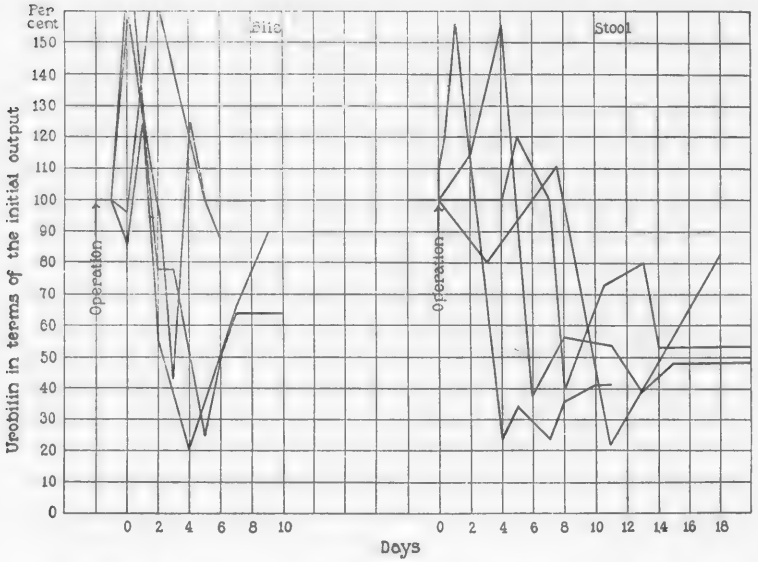
When the two cannulas had been placed they were tied together to avoid stoppage of bile flow by angulation of them.

Previous work from this laboratory has shown that samples of bile taken at the same time from different regions of the liver, have approximately the same volume per gm. of liver and contain equal amounts of bile pigment per cc.[18] Knowing then the amount of bile and the bilirubin content in the daily sample yielded to the collecting apparatus, and, finding at autopsy, by injection methods, the exact amounts of liver tissue secreting into the balloon and into the intestine, respectively, the amount of bilirubin and bile put out by the entire liver on any given day can be estimated and so too with that portion entering the intestine.

Animals that underwent merely a sampling intubation, after the manner described, with result that most of the bile still flowed into the intestine, showed urobilin in the stool and bile at all times. Text-fig. 3, which depicts the urobilin findings in the bile and feces of four animals so treated, in which on the average 25 per cent of the bile was lost as a sample, should be contrasted with Text-fig. 1, of the dogs intubated for the collection of total bile. In this chart as in the previous one the urobilin output in the bile of the 1st day after operation and the average finding in the feces before operation are arbitrarily plotted as 100 per cent. Instead of the progressive fall in the urobilin of the bile that occurred with total bile deprivation, these sampled dogs showed first an increase in the pigment, then a fall, and by the 6th day after operation some return toward the initial amount. The early increase in the urobilin of the bile occurred at the time when the greatest amount of bilirubin was being secreted, that is to say, at the time when the largest amounts of this pigment were entering the intestine. For the bilirubin output of these dogs, like that of the common duct intubation animals,[3] was greatest shortly after operation. That the increased amount of urobilin in the bile is not, like that of the bili-

rubin, due to the absorption of blood lost during the operation is shown by the fact that the urobilin of the bile steadily decreases following the operation to drain the total bile by intubation of the common duct (Text-fig. 1).

In the eleven animals intubated for the collection of partial bile the normal fecal output of urobilin was determined daily in some cases



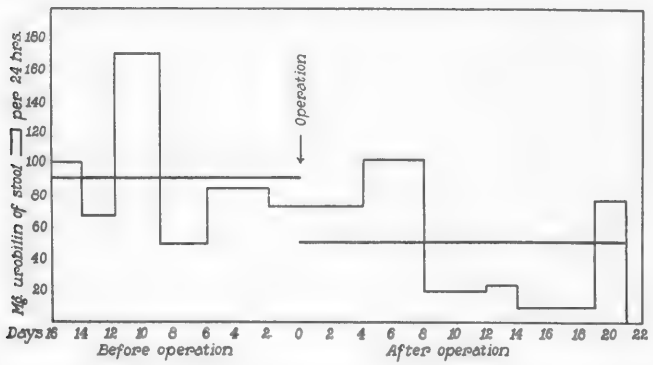
TEXT-FIG. 3. Continued presence of urobilin in both bile and feces after intubation for the collection of bile samples only.

Text-fig. 3. Each line represents the findings in a dog. More than 70 per cent of the biliary secretion of these animals entered the intestine, the remainder being collected for analysis. Urobilin was constantly present in both bile and feces.

for several weeks prior to the intubation. After it there generally occurred in the stool, as in the bile, an increase of urobilin from the 3rd till the 6th day, then an abrupt fall, followed by a rise again toward the normal preoperative output. It is significant that a complete return to this output did not occur even in experiments of long duration. The decrease in the stool urobilin roughly corresponded with

the percentage of the total lost to the body in the sample. The correspondence was only approximate at best for variations in the intestinal motility from day to day can greatly change the urobilin content of the stool.

Text-fig. 4 shows the urobilin of the feces, before and after intubation for the collection of but 32 per cent of the total bile. The heavy horizontal lines show the average urobilin content of the feces before and after operation.



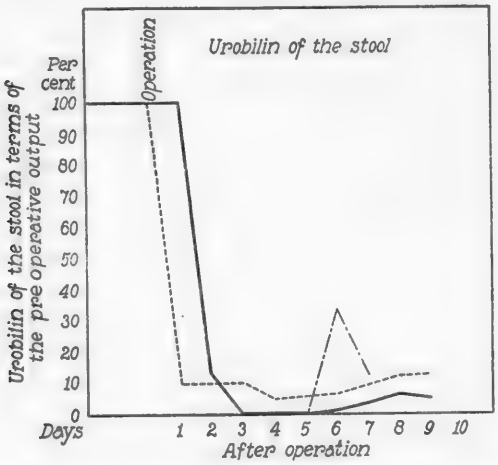
TEXT-FIG. 4. Decrease in the urobilin of the feces after intubation for the drainage of 32 per cent of the total bile.

Text-fig. 4. In this animal the bile duct to a single liver lobe (32 per cent of the whole) was intubated with result in the daily loss of this amount of the secretion. It will be seen that the decrease in the urobilin is roughly proportional to the percentage of the total bile lost. The heavy horizontal lines show the average urobilin content of the feces before and after operation. With the withdrawal of 32 per cent of the bile the urobilin of the feces fell 43 per cent.

The Urobilin Changes on Total Biliary Obstruction.

The effect of complete exclusion of bile from the gastrointestinal tract but without its removal from the body was studied in instances of total biliary obstruction produced by severance of the common duct between ligatures. Only those instances will be considered in which complete occlusion of the biliary system was maintained throughout the observations as proven at autopsy by injection methods and dissection. There were three such animals out of seven operated

upon. All became heavily jaundiced, yet the urobilin of the stool fell off quite as rapidly as in the dogs from which the total bile was drained (Text-fig. 5). The feces of one became entirely urobilin-free and in the other two instances almost so, but by the 5th day after operation, when jaundice was well developed, appreciable amounts of the pigment, more than mere traces, had reappeared in the stools. At autopsy the intestinal mucosa of the totally obstructed animals was in-



TEXT-FIG. 5. The urobilin content of the stools after total biliary obstruction.

Text-fig. 5. Plotted like the previous ones, but for the stool findings alone to show the changes in amount of the fecal urobilin of animals undergoing a total biliary obstruction. The urobilin of the stool fell off quite as rapidly as in instances in which the animals were deprived of all the bile by intubation of the common duct (Text-fig. 1) but small amounts of the pigment reappeared in the feces by the 6th day after operation. The continuous, broken, and dotted lines each represent the findings in a different individual.

tensely yellow and gave the reaction for bile pigments strongly. It seems probable, then, that during jaundice, small quantities of bilirubin escape into the lumen of the intestine and are there changed to urobilin. Possibly this mechanism accounts for the very slight traces of the pigment found in the feces of animals deprived of the total bile. As will be seen later, small or large quantities of blood in

the intestine in the absence of bile, cannot account for the presence of this pigment.

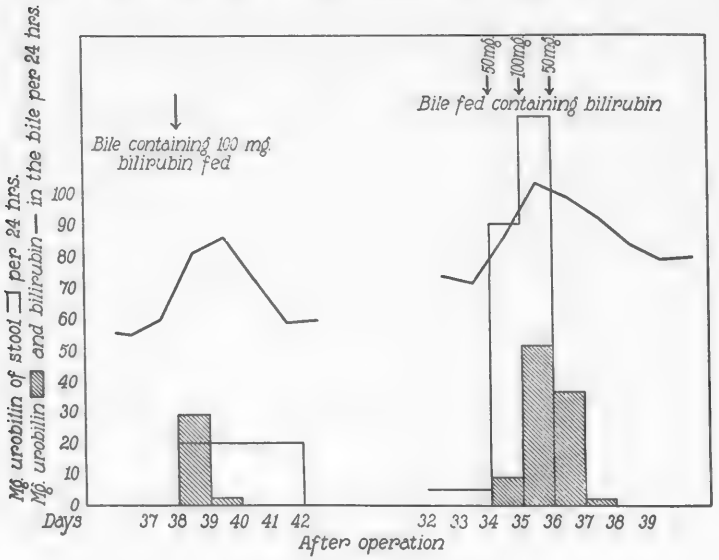
The urinary findings possess much interest. After the operation for total biliary obstruction, urobilin appeared in the urine for 1 or 2 days in moderate quantities, but then disappeared, not to be found again. The following experiment, with others like it, to be reported in full later on, have shown that the practically complete lack of urobilin in the excreta of animals with total biliary obstruction is not to be explained as the result of changed conditions within the bowel, as distinct from an absence of bilirubin. For when bilirubin is fed as fistula bile free from urobilin to animals with biliary obstruction urobilin appears in quantity in both the stools and urine.

A dog in which total biliary obstruction was produced yielded, 3 days afterwards, feces and urine that were both urobilin-free. The stools of the 6th day contained small amounts of the pigment, less than 1 per cent of that found prior to operation. Up to this time there was no urobilinuria. Fresh sterile dog bile from another animal, containing 100 mg. of bilirubin and no urobilin was fed by stomach tube on 2 successive days. There followed at once a great rise in the intestinal urobilin to the original value, intense urobilinuria, and an increase in the bilirubinuria. The two latter phenomena were transient but the high fecal urobilin findings persisted for nearly a week with a gradual fall to the amount obtained before the feedings.

The Effect on the Urobilin Output of Bile Feedings.

In our experiments with vigorous dogs losing the bile as result of intubation urobilin was never found in this secretion save when there had been opportunity for bile pigment to reach the intestine in some way. There are three conceivable sources for the urobilin present under these latter circumstances. It may be formed as such by the liver and secreted into the bile, it may arise in other tissues and be brought to the liver in the blood, or it may occur in consequence of a reabsorption of urobilin from the intestine with its removal from the blood and secretion into the bile by the liver. The disappearance of the pigment from both bile and stool when bile is diverted from the intestine furnishes strong support for the latter explanation which is convincingly demonstrated to be the true one by experiments in which urobilin reappeared in the bile of completely intubated bile fistula animals, fed urobilin-free bile by stomach tube.

Eight dogs with intubated common duct, all with bile, urine, and feces free from urobilin, were fed by gavage sterile urobilin-free dog bile. At times the material came from the animals themselves and again from others. In every instance of such feeding the bile collected during the next 24 hours contained urobilin in greater or less



TEXT-FIG. 6. The effect of bile feedings on the urobilin output.

Text-fig. 6. A chart showing the effects of bile feedings to two animals that were urobilin-free as a result of the intubation of the common duct. The sudden appearance of urobilin in the bile collected during the 24 hours after the feedings and its disappearance 2 days after the last one, are shown. The bilirubin output in the bile became notably greater after the bile feedings but returned to the previous level when these were discontinued. The changes are typical of those observed in eight such experiments.

amount. When the feedings were repeated for many days this pigment continued to appear throughout the period, and, persisted in the bile for 24 or 48 hours after they had been stopped. Text-fig. 6 gives the data for two such animals.

Dog A, 9 kilos, in excellent condition, was yielding, on the 38th day after intubation of the common duct, sterile bile containing no urobilin.

Day after day the bilirubin output had been uniform, on the 36th to 38th days, 55.1, 54.3, and 59.6 mg., respectively, of this pigment being eliminated in the bile. Now 128 cc. of urobilin-free bile from another dog, containing 100 mg. of bilirubin was given by mouth. The bile of the next 24 hours contained 30 mg. of urobilin and the bilirubin had increased from 59.6 to 81 mg. The following day, with no further bile feedings, a smaller amount of urobilin was eliminated, 2.7 mg. but more bilirubin, 85.3 mg. A day later a rapid fall to the normal bilirubin level took place with but a trace of urobilin present, and none 24 hours later.

Dog B, 15¾ kilos, in excellent condition after intubation of the common duct and on the diet of 1,050 gm. of bread, wet with milk and 450 gm. lean meat, yielded sterile bile, urobilin-free and of uniform bilirubin content. The stools and urine were likewise urobilin-free. On the 33rd and 34th days of the experiment, respectively, the bile contained 73.3 and 71.0 mg. of bile pigment. At this time, 34 days after operation, 104 cc. of sterile urobilin-free bile containing in all only 50.0 mg. of bilirubin was given by mouth. The hepatic bile of the next 24 hours showed 9.0 mg. of urobilin and 85.3 mg. of bilirubin, an increase in the latter pigment of 14.3 mg. Urobilin was now present in the feces in great amounts. A second bile portion containing 100.0 mg. of bilirubin and no urobilin was then fed; and the bile of the next 24 hours contained 51.0 mg. of urobilin and 102.8 mg. of bilirubin. In the stool the preoperative amount of urobilin was found and, in the urine a small quantity. Text-fig. 6 shows that after a further smaller feeding of urobilin-free bile, containing 50.0 mg. of bilirubin, 36.0 mg. of urobilin was eliminated in the hepatic bile of the next day, with 98.4 mg. of bilirubin. Bile feedings were then discontinued. Within 48 hours the bilirubin output had fallen to the usual level and the urobilin had disappeared.

In all, twelve bile feeding experiments were made upon separate occasions to nine of the dogs with common duct intubation, all of which had been yielding bile free from urobilin. In some instances a single feeding was given, in others several on successive days. In every case urobilin appeared thereafter in the fistula bile. Bile fed by mouth is subjected to gastric digestion and may be changed in some manner thereby. To avoid this difficulty as affecting the observations, a type of intubation was devised whereby, at will, all of the animal's bile could be collected or allowed to flow into the intestine through the papilla of Vater.

Urobilin as Affected by Intermittent Bile Loss.

Modifications of the ordinary bile fistula have often been attempted. As early as 1868[25] and 1870[27] Schiff described a cannulation of the

[26] Schiff, M., *Gior. Sc. naturali ed economiche*, 1866, iv (cited in Schiff[27]).
[27] Schiff, M., *Arch. ges. Physiol.*, 1870, iii, 598.

gall bladder which permitted of the collection of bile from that organ at will. Since the secretion had access to the ducts as well as to the gall bladder, the quantity of bile obtained depended upon the functioning of the sphincter of Oddi for bile flowed into the intestine when this relaxed. It was assumed by Schiff that in the digestive interim between feedings, bile was retained in the duct system and could be collected from the gall bladder. He termed this an "amphibilous" fistula.

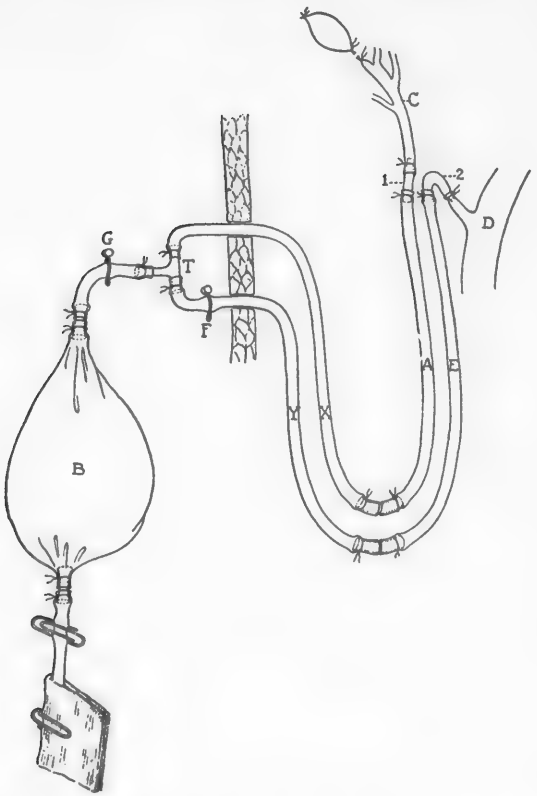
For us it was essential to obtain whenever desired, a complete bile deprivation or a passage of the secretion to the intestine. The type of intubation eventually adopted whereby the bile may flow either into a collection bag or into the intestine, but not into both, we will term an "altercursive intubation."

"Altercursive Intubation."

The intubation is illustrated in Text-fig. 7. The common duct was joined to two cannulas *1*, draining from the liver and *2* directed toward the intestine, each with a soft rubber connection (*A*, *E*). To these latter, tubes of firmer rubber (*X*, *Y*) were jointed which led outside the body through punctured orifices, and ultimately to the collecting balloon. The bile could either be collected, by clamping the tubing at the point *F*, or be shunted back to the lower segment of the common duct by clamping at *G*. Under this latter arrangement the secretion simply made the passage of the tubing and reentered the common duct at a point not over 1 cm. from that at which it had left it. The sphincter of Oddi was not affected by this procedure, for the lower cannula was placed $1\frac{1}{2}$ to 2 cm. above it.

This type of intubation proved highly successful, infection of the bile occurring seldom, despite the foreign system through which the secretion flowed. During the period of collection to the balloon, that is to say for several days often, bile remained stagnant within the efferent tubing and the lower portion of the common duct. In spite of this no ascending infection of the bile or tubing occurred. Evidently the physiological mechanism to prevent the passage of bacteria above the sphincter of Oddi is highly effective.

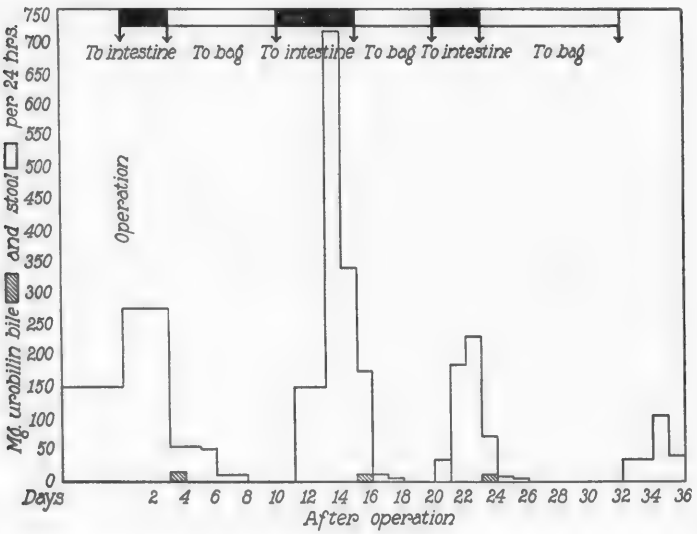
Nine dogs intubated successfully were used. During the first days after laparotomy, when extravasated blood was being absorbed



TEXT-FIG. 7. Diagram of the "altercursive intubation."

Text-fig. 7. The cannula *1* was placed in the common duct well below the entrance of the duct from the caudate and right lateral lobes. The bile flowed out the afferent tube *A* to a glass T-tube which led both to the collecting balloon *B* and to the efferent tube *E*. By clamping at *F* or *G* the bile could be shunted at will to the intestine or to the balloon. All collections were made under aseptic conditions as described in a previous paper from this laboratory.[2] *A*, afferent soft rubber tube; *E*, efferent soft rubber tube; *X*, afferent hard rubber tube; *Y*, efferent hard rubber tube; *C*, common duct; *D*, duodenum; *F*, *G*, clamps; *T*, T-tube; *B*, collecting bag.

bile was allowed to flow uninterruptedly, through the tubing into the intestine. The fecal urobilin after the usual postoperative rise, soon fell to the preoperative level. Later, for a number of days, the return flow of bile to the intestine was prevented and all of the secretion was collected into the balloon, just as in a common duct intubation animal. Outspoken urobilin changes took place as a result of these procedures. The typical findings as illustrated in a single animal are depicted in Text-fig. 8. They may be summarized as follows, for the group:



TEXT-FIG. 8. The urobilin changes caused by intermittent diversion of the bile stream from the intestine.

Text-fig. 8. For 3 days after the operation for "altercursive intubation" the bile flowed into the intestine. The stream was then diverted to the collecting balloon. Urobilin was present in the bile collected during the first 24 hours but not thereafter. After the animal had been losing bile for 8 days urobilin could no longer be demonstrated in the stool. On the 11th day of intubation the bile was again allowed to flow into the intestine. Practically at once urobilin appeared in the feces. The further observations of like sort have been charted. Each time the bile stream was diverted or again directed to the intestine the sequence of urobilin changes was the same.

During the first period of the intubation no bile was obtained for study, of course. That first collected when the secretion was diverted to the balloon contained urobilin. Thereafter its amount rapidly decreased though some was usually to be found for 1 or 2 days. Later none was ever present. The urobilin of the feces disappeared at about the same time as that of the bile and in both respects the animals came to resemble dogs deprived of the total bile—which indeed they were. After nearly a week the bile was allowed to flow once more to the intestine. In 1 or 2 days urobilin reappeared in the stool and continued to be present there in normal amounts. When, later, the total bile was again taken into the balloon and excluded from the intestine the same sequence of events occurred. The intermittent diversion of the bile was repeated many times in each instance, always with the same, highly characteristic result.

In passing it is of interest to note that when bile is taken daily in the collecting balloon, following a period in which it has been allowed to flow into the intestine, the amount of it, and of bilirubin as well, is at first relatively large compared with that obtained some days later. The decrease is gradual and ordinarily a constant level is reached after about 6 or 8 days of bile deprivation, a point which is not shown in the chart. The significance of this finding for the better understanding of the physiology of bile volume and bile pigment output, is an obvious one, to be taken up in detail in a subsequent paper.

Urobilin Is not Directly Derived from Bleeding into the Bowel or Tissues.

From these experiments it would seem that the presence of urobilin in the body is directly dependent upon the entrance of bile or bile pigments into the intestine. But may it not sometimes be derived from free hemoglobin especially when present in the gastrointestinal tract? Some investigators have assumed that blood pigment becomes changed to urobilin in the intestine; and workers supporting the histogenous hypothesis of urobilin origin consider it possible for various cells of the body to convert the hemoglobin liberated in interstitial hemorrhage, to this pigment directly, [28,29] or so to change it that a urobilinuria results from absorption of the products.[30]

[28] Quincke, H., *Virchows Arch. path. Anat. u. Physiol.*, 1884, xcv, 125.
[29] Brulé, M., Recherches récentes sur les ictères, Paris, 1919.
[30] Kunkel, A., *Virchows Arch. path. Anat. u. Physiol.*, 1880, lxxix, 455.

In our experiments it was frequently noticed that the stools of animals intubated for the collection of total bile, though acholic and urobilin-free, were often tinged with blood and gave a positive guaiac test, at times when the exceedingly sensitive test for urobilin was negative. This was observed at times in six or seven out of fourteen dogs studied. At autopsy the affected animals showed slight hemorrhagic injection of portions of the intestinal mucosa.

To study the relation of the presence of hemoglobin in the intestine to urobilin in feces and bile, two dogs with the common duct intubated and two others which were being deprived of their bile after "altercursive intubation," all yielding urobilin-free bile and stools, were fed dog and ox blood in varying amounts, 25 to 100 cc. for 4 days. In the cases of dogs with the "altercursive intubation," the blood was injected directly into the duodenum through the efferent tube or fed by mouth on 4 successive days. The stools of these animals soon became black and tarry, and gave exceedingly strong guaiac tests but did not at any time contain urobilin. The bile too remained urobilin-free and the 24 hour bilirubin output in it remained unchanged throughout the experiment.

In the past authors have reported increases in the urobilin of the bile or the appearance of urobilinuria following interstitial,[30] and even intracranial[31] hemorrhage. The increase in the pigment has been taken to indicate a formation of urobilin directly in those tissues, its passage into the blood and its secretion by liver and kidneys into the bile and urine.

Experiments to be detailed in full later, show clearly that no urobilin is to be found under conditions involving blood destruction when all bile pigment is excluded from the intestine. Briefly, in animals intubated for the collection of total bile, marked hemolysis was produced by the introduction of distilled water, by withdrawing 200 to 300 cc. of blood, laking it with water, and injecting the centrifugalized hemolyzed fluid intravenously, and by feedings of toluylenediamine. In three other instances large hematomas were produced. The animals used were receiving no bile pigment in the intestinal tract, the bile, feces, and urine were urobilin-free, and the bilirubin content of the

[31] von Bergman, E., in Volkmann, Sammlung klinische Vortr ge, Berlin, 1881, No. 190 (Surg. No. 57), 1558, 1560.

bile was uniform from day to day. After the hemolysis and interstitial hemorrhage no urobilin was found at any time in the excreta of any of these dogs. Enormous increases in the bilirubin elimination of the bile gave proof of the reaction of the liver to the masses of hemoglobin liberated in the body by both procedures. At times the hemolysis was sufficiently severe to cause pronounced hemoglobinuria but no traces of urobilin appeared in any of the excreta.

When similar experiments were done upon animals intubated for the collection of part of the bile only, so that the greater part of the bilirubin still reached the intestine, the large increases in the bilirubin content of the bile after the liberation of hemoglobin, were followed by, or often synchronous with, increases in the urobilin of bile and stool and at times a urobilinuria occurred. All these changes are plainly attributable to the increased amounts of bilirubin entering the intestine, there to be converted to urobilin.

In this paper it has been our intention to consider chiefly the normal physiology of urobilin. The findings just mentioned, relative to the effects of hemorrhage and blood destruction upon the pigment, will be reported more fully later.

DISCUSSION.

These experiments have yielded answers to a number of questions of prime importance in urobilin physiology. Together they prove that the existence of urobilin in the stool, and in the bile as well, under normal conditions depends on a delivery of the bile itself, or, to speak more precisely, of bilirubin to the intestine.

The earlier work of others has established the fact that urobilin can be obtained from bilirubin, *in vitro*, by reduction.[10,11] That this change takes place in the intestine as well, by the action of bacteria, has also long been known. In recent years[12] experiments upon the method whereby this ordinarily comes about in man have shown that certain of the intestinal flora, especially *Bacillus putrificus* and other bacteria possess this transforming ability.

The findings reported in the present paper point directly to an absorption of urobilin from the intestine and its secretion into the bile by the liver. An enterohepatic circulation of bile pigment has been demonstrated by previous work from this laboratory.[6]

SUMMARY.

Methods have been developed whereby animals can be totally, partially, and intermittently deprived of their bile, without infection of this secretion or of the duct system. In all the instances considered the bile remained sterile throughout the period of the experiment. With this aid we have studied urobilin physiology.

We have been able to show that the normal presence of urobilin in the bile and feces of dogs depends on the passage of bile pigment to the intestine, either through the normal channels, or by abnormal ones, as when it is fed by mouth. Complete loss of the bile from the body resulted in the total disappearance of urobilin and urobilinogen from the bile, feces, and urine. Rarely very faint traces remained in the feces, the origin of which has been discussed. Partial loss of the bile resulted in a corresponding reduction in the urobilin of the dejecta.

Feedings of sterile urobilin-free dog bile to intubated dogs losing all of their bile and having no urobilin in it, or in feces or urine, were followed by the appearance of the pigment in the hepatic bile secreted shortly thereafter. When the feedings were stopped the urobilin soon disappeared.

Total obstruction of the bile flow caused disappearance of the urobilin of the bile and stool. Later as the animals became heavily jaundiced the pigment appeared again in very small quantity in the feces. Autopsy at this time showed that the intestinal mucosa was deeply tinted with bilirubin some of which undoubtedly had passed into the lumen of the bowel and had there been changed to urobilin.

Employment of the "altercursive intubation," by which an intermittent diversion of the bile stream of animals from the intestine to a collecting apparatus could be effected, showed that while bile pigment still reached the intestine urobilin was present in the bile secreted by the liver but that almost at once after the bile had been diverted from the gut urobilin disappeared from it. In relation to this finding it was noticed that in animals from which merely a small fraction of the bile was collected, that from a single liver lobe, while the greater portion reached the intestine there was most urobilin in the bile at times when most bilirubin was entering the intestine.

STUDIES ON THE OXIDATION-REDUCTION OF HEMOGLOBIN AND METHEMOGLOBIN.

II. The Oxidation of Hemoglobin and Reduction of Methemoglobin by Anaerobic Bacilli and by Sterile Plant Tissue.

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INTRODUCTION.

In the present paper certain principles involved in the operation of the oxidation-reduction systems of anaerobic bacilli are illustrated by the action of these bacteria upon oxyhemoglobin, hemoglobin, and methemoglobin. Certain relations and differences between the oxidizing-reducing action of anaerobes and of pneumococci are evidenced by a comparison of the action of these two groups of bacteria upon blood pigments. The oxidizing-reducing action of plant tissue is illustrated by experiments on the oxidation-reduction of the blood pigments by the sterile potato.

Now that the physical-chemical relations between oxyhemoglobin, hemoglobin, and methemoglobin have been made more clear by the recent study of Conant (1) it is possible to illustrate microbial oxidation-reductions by the action of bacteria upon these blood pigments (2). The conversion of oxyhemoglobin to hemoglobin is a deoxygenation and in a closed system may be used as a measure of the consumption of molecular oxygen. The conversion of hemoglobin to methemoglobin is a true oxidation in the electronic sense and it seems that this reaction, under defined conditions, may be interpreted as an index of the formation of oxidizing agents. Since methemoglobin is changed to hemoglobin in systems of sufficient reducing power, this reaction may be used as a measure of reduction activity. All of these reactions may be followed quantitatively by the refined gasometric methods employed in blood gas analyses.

In the preceding paper (2) the oxidizing-reducing activities of Pneumococcus were demonstrated by the changes induced in blood pigments. An investigation on the action of anaerobic bacilli upon the blood pigments should likewise reveal

some of the principles governing the operating of the oxidation-reduction systems of anaerobes. Oxidation-reduction systems of the same general type as those found in Pneumococcus probably constitute a part of the total oxidizing-reducing equipment of all bacterial cells. In the case of anaerobic bacteria, the conditions limiting the operation of the oxidation-reduction systems of the cell seem to be similar in nature to those obtaining for Pneumococcus. Both anaerobes and pneumococci can grow at greatly reduced oxygen tensions or in fact in the complete absence of molecular oxygen. Moreover, neither of these groups of bacteria is equipped with the enzyme catalase which is commonly present in almost all microorganisms. A further relation is suggested by the fact that exposure to air, which is known to be toxic to many of the strict anaerobes, also results in the inactivation of enzymes and other constituents which are involved in the life and growth of pneumococci (3, 4).

Although much valuable knowledge concerning the growth of anaerobes has accumulated, the whole question of anaerobiosis is still open. The subject has recently been presented from a broad point of view in a review by Clark (5). Two facts, however, are established: that anaerobes can grow in the complete absence of molecular oxygen, and that exposure to molecular oxygen proves definitely toxic to some at least of the more strict anaerobic bacteria. In explanation of this latter phenomenon Avery and Morgan (6) have suggested that the toxic effect of exposure to air upon anaerobes is due to the action of peroxides upon the peroxide-sensitive anaerobic organism. Similar views have been expressed by McLeod and Gordon (7) and by Callow (8). Anaerobes are apparently devoid of catalase and peroxidase enzymes[1] and moreover, the anaerobic cell which in its usual environment does not encounter peroxides may be especially sensitive to such substances. In support of their theory, Avery and Morgan (6) have grown strict anaerobic bacilli in media fully exposed to air, but which contained substances possessing a "peroxidase" action. If such substances are not present, not only do the anaerobes fail to grow in the medium but they are rapidly killed.

EXPERIMENTAL.

Methods.—The present study has been made with strains of *B. histolyticus* and *B. aerofœtidis*. The strains used are strict anaerobes. Inocula of 0.5 cc. of a young, actively growing culture of either of these strains fail to grow and ultimately die when introduced into 5 cc. of meat infusion broth in an ordinary culture tube. The cell suspensions used in the following experiments were prepared by centrifuging young, actively growing anaerobic cultures (5 to 8 hours),

[1] It has been observed by Avery and Morgan (unpublished experiments) that Pneumococcus also contains much less peroxidase than do most bacteria. We have made similar observations of a lack of peroxidase in the cells of a number of anaerobic bacilli.

which by repeated transfers in broth at short intervals had been rendered practically free from spores. Precautions were taken during the preparation of the cell suspensions to reduce exposure to air to a minimum.

Analytical Methods.—The "oxygen content" analyses and hemoglobin determinations by "oxygen capacity" were made by the methods described by Van Slyke and Neill (9). The determinations of hemoglobin by "carbon monoxide capacity" were made by the procedure described by Harington and Van Slyke (10). "Total pigment" (HbO_2 + Hb + MetHb) determinations were made by Stadie's colorimetric method (11).

Owing to the marked oxygen-consuming powers of anaerobic cells, considerable care was necessary to obtain satisfactory results in the determinations of the "oxygen capacity" of hemoglobin solutions containing large numbers of anaerobic bacteria. Satisfactory results were obtained by making the saturations at low temperatures (0–5°C.) and rapidly transferring the oxygenated hemoglobin to the analysis pipette of the apparatus. Most vigorous aeration and rapid admission of the sample are necessary to maintain complete saturations. Both theoretically and practically, "carbon monoxide capacities" are to be preferred to "oxygen capacities" as measurements of hemoglobin under these conditions.

Consumption of Molecular Oxygen and Establishment of Reducing Conditions by Anaerobic Bacilli.

It is a common laboratory observation that if very large numbers of anaerobic bacteria are taken from an anaerobic culture at the height of its activity, growth may be induced in ordinary culture media without any precautions for the exclusion of air. Under the same conditions smaller inocula fail to grow. This phenomenon seems to be explained by the fact, pointed out in Clark's review (5), that actively growing anaerobic cells, if not overtaxed, can establish their own reducing conditions even in the presence of a certain amount of molecular oxygen.[2] These relations can be illustrated by the following experiments, which demonstrate the rapid consumption of molecular oxygen and the establishment of reducing conditions by actively growing anaerobic bacteria.

[2] The addition of a large number of bacterial cells to culture media furnishes a system of considerable oxygen-consuming and reducing power. Unless the liquid is vigorously and constantly agitated with shallow layers exposed to the air, it is probable that the culture is never exposed to the oxygen tension of the atmosphere.

In the typical experiment reported below, a suspension of anaerobic bacilli was added to a tube containing oxygenated laked blood. In such a system, the anaerobes are confronted not only with the usual amount of molecular oxygen dissolved at atmospheric tension, but with a considerable reserve of molecular oxygen which is dissociated from the oxyhemoglobin upon the consumption of the dissolved oxygen. The system containing this reserve oxygen supply was sealed after the addition of the anaerobic bacilli. Subsequent determinations of the "oxygen content" of the system were made to test the consumption of the molecular oxygen; gasometric hemoglobin analyses ("oxygen capacities") were made to determine if any hemoglobin was destroyed by products formed in the oxygen-consuming reactions.

The results of a typical experiment are tabulated in Table I.

TABLE I.

Consumption of Molecular Oxygen and Establishment of Reducing Conditions by Anaerobic Bacilli.

Reaction mixture.	Original.		After 2 hrs. at 37°C.		Oxygen consumed.	Hemoglobin oxidized to methemoglobin.
	O_2 content ($HbO_2 + O_2$).	Ferrous hemoglobin ($HbO_2 + Hb$).	O_2 content ($HbO_2 + O_2$).	Ferrous hemoglobin ($HbO_2 + Hb$).		
	mM	mM	mM	mM	mM	
3 cc. suspension of anaerobic cells (*B. histolyticus*) + 6 cc. sterile laked blood.	4.09	3.80	0.0	3.75	4.09	—
3 cc. broth + 6 cc. sterile laked blood.	4.09	3.80	4.00	3.83	0.09	—

As evidenced in Table I, the anaerobes consume every detectable trace of molecular oxygen and soon exhaust the reserve oxygen furnished by the oxygen dissociation of oxyhemoglobin. If young, actively growing cells are used, all of the oxyhemoglobin is deoxygenated with surprising rapidity. Naturally enough, there is a limit to the "capacity" of the anaerobic cells to establish reducing conditions in the presence of a reserve supply of molecular oxygen. The reducing action is resident in the cells, and, in experiments in which small numbers of cells are introduced into systems containing a large amount of reserve oxygen, all of the oxyhemoglobin is not deoxygenated.

Reduction of Methemoglobin by Anaerobic Bacilli.

In the experiments for which the protocol given in Table II serves as an example, suspensions of anaerobes were added to solutions of methemoglobin. As indicated in the preceding experiment, the anaerobes can establish their own reducing conditions in systems containing to begin with a considerable supply of molecular oxygen. Hence, it is not necessary to use methemoglobin solutions entirely free from oxyhemoglobin. Results similar to those given in Table

TABLE II.

Reduction of Methemoglobin by Anaerobes.

Reaction mixture.	Original.				After 2 hrs. at 37°C.			
	Total hemoglobin (MetHb + HbO_2 + Hb).	O_2 content (HbO_2 + O_2).	Ferrous hemoglobin (HbO_2 + Hb).	Ferric hemoglobin (MetHb).	O_2 content (HbO_2 + O_2).	Ferrous hemoglobin (HbO_2 + Hb).	Ferric hemoglobin (MetHb).*	Methemoglobin reduced to hemoglobin.
	mM	mM	mM	mM	mM	mM	mM	mM
10 cc. methemoglobin solution (mixture HbO_2, Hb, and MetHb) + 5 cc. suspension of anaerobic bacilli (*B. histolyticus*).	4.1	1.0	0.7	3.4	0.0	3.8	0.3	3.1
Control. 5 cc. methemoglobin solution + 2.5 cc. broth.	4.1	1.0	0.7	3.4	0.9	0.8	3.3	—

* Total hemoglobin minus ferrous hemoglobin.

II are obtained in mixtures of oxyhemoglobin and methemoglobin, provided active cells in sufficient numbers are used and the system sealed to prevent continued access of air.

The data presented in Table II illustrate the reduction of methemoglobin to hemoglobin by the reducing activity of anaerobic bacilli. The hemoglobin formed from the methemoglobin may be determined gasometrically by converting it either to oxyhemoglobin or to carbon monoxide hemoglobin.

The methemoglobin which was used in the preceding experiments

to illustrate the reducing action of anaerobes was a product formed by the spontaneous alteration of sterile hemoglobin. Similar results have been obtained in experiments in which the methemoglobin was formed by the oxidizing activity of pneumococci. Suspensions of *Bacillus coli* can likewise serve as reducing agents in the transformation of methemoglobin to hemoglobin.

Influence of Oxygen Tension upon the Oxidation-Reduction of Hemoglobin and Methemoglobin by Anaerobic Bacilli.

Owing to the pronounced reducing activity of the anaerobes, a mixture of the bacilli and hemoglobin can be maintained at a definite tension of molecular oxygen only in a system which is kept constantly saturated with a known gas mixture. With the cooperation of Dr. A. Baird Hastings, a number of experiments have been made in which mixtures of anaerobes and hemoglobin were maintained at known tensions of molecular oxygen. This was accomplished by continued saturation in large tonometers, with the technique employed in the study of gas and electrolyte equilibria of the blood (16). The data obtained in one of these experiments are presented in Table III.

Complete details of the procedure will be given in a subsequent paper. Aseptic technique was used throughout. Under these conditions it was possible to carry through without contamination the preliminary saturation, admission of anaerobes to tonometers, and the final saturation up to the point of actual analysis at the end of the experiment. Subcultures were made on all mixtures just before analysis and they proved absolutely free of aerobic microorganisms.

The results presented in Table III demonstrate that significant amounts of methemoglobin are formed in a mixture of hemoglobin and anaerobic bacilli if the mixture is maintained at the optimum oxygen tension for the reaction. It is interesting to observe that anaerobic bacilli which induce such strong reducing conditions in the absence of molecular oxygen, do oxidize hemoglobin to methemoglobin in the presence of molecular oxygen. Apparently, the optimum conditions for the oxidation of hemoglobin by anaerobes are the same as those previously shown for pneumococci. These conditions are fulfilled only when the mixtures are constantly saturated at oxygen tensions sufficiently high to cause the formation of oxi-

dizing agents and to mask the potential bacterial reducing activity, and yet sufficiently low to permit the oxygen dissociation of a large proportion of the hemoglobin. It is probable that most if not all of the hemoglobin destroyed was oxidized to methemoglobin as the presence of a considerable amount of this pigment was confirmed by spectroscopic examination. That no methemoglobin was formed in the test carried out in the absence of oxygen was to have been expected.

In the preceding paper it was shown that the ratio between hemoglobin and methemoglobin which is established in a mixture of blood and pneumococci may be altered in either direction by changes in the tension of molecular oxygen. Similarly, when hemoglobin which has been formed by the reducing action of anaerobic bacteria upon methemoglobin is subsequently freely exposed to air, part of the

TABLE III.

Influence of Oxygen Tension upon the Oxidation of Hemoglobin by Anaerobic Bacilli.

Oxygen tension.	Oxygen dissociation of oxyhemoglobin.		$HbO_2 + Hb$	MetHb formed.
	HbO_2	Hb		
mm.	*per cent*	*per cent*	mM	mM
0	0	100	2.87	0.0
20	50	50	2.30	0.6
710	100	0	2.65	0.2
Control.	—	—	2.85	—

hemoglobin is reconverted to methemoglobin. The reversal of the reaction in this instance may be explained by the fact that the anaerobic bacteria possess both oxidizing and reducing power, as do pneumococci, and hence can either oxidize or reduce the blood pigments, depending upon the presence or absence of molecular oxygen.

Activation of the Reducing and Oxygen-Consuming Powers of Washed Anaerobic Bacilli.

In previous papers it has been shown that washed pneumococcus cells by themselves cannot establish reducing conditions or form oxidizing agents (2, 15, 17–19). Thus, washed pneumococci or saline extracts of them can neither reduce methemoglobin and methylene blue nor oxidize hemoglobin until the "incomplete" oxidation-reduction systems of the washed cells are "completed" by the

addition of certain substances (meat infusion, yeast extract, etc.). Similar relations have been reported for other bacteria by Harden and Zilva (20) and by Gillespie (12).

Experiments were made to determine if the washed cells of anaerobic bacteria by themselves can consume oxygen and establish reducing conditions, or if the washed cells require the presence of other substances in order to exhibit these activities. These experiments were similar in nature to those previously made with the washed cells of Pneumococcus. Tests were made of the reducing and oxygen-consuming powers of suspensions of the washed cells alone, and of mixtures of different substances (glucose, meat infusion, etc.) with unheated and heated suspensions of the washed bacteria.

The establishment of reducing conditions was determined by the use of methylene blue as well as methemoglobin. A series was included in which the deoxygenation of oxyhemoglobin served as an indicator of the ability of the different mixtures to consume molecular oxygen. The various substances added to the washed cells included glucose, meat infusion, alcoholic extract of potato juice, and alcoholic extract of meat infusion.

Suspension of Washed Anaerobic Cells.—A 9 hour culture of *B. histolyticus* was centrifuged; the cells were washed in sterile salt solution and suspended in sterile 0.1 M phosphate solution (pH 7.5). One-half of the suspension was heated 10 minutes at 100°C. to inactivate thermolabile cellular substances involved in the oxidation-reduction systems of the anaerobes.

Substances Added to the Washed Cells.—Glucose: sterile 2 per cent glucose in 0.1 M phosphate solution (pH 7.5). *Meat Infusion:* meat infusion prepared as described in a preceding paper (19), adjusted to pH 7.5 before use.

Alcoholic Extract of Potato Juice.—Freshly expressed potato juice was extracted in six times its volume of redistilled alcohol. The alcoholic extract was concentrated almost to dryness at 50°C. *in vacuo* and extracted again in 95 per cent alcohol. The second alcoholic extract was concentrated to dryness *in vacuo* and the residue was taken up in sterile phosphate solution (pH 7.5) to a volume equal to twice that of the original potato juice. Precautions were taken to prevent oxidation throughout the procedure.

Alcoholic Extract of Meat Infusion.—Meat infusion was concentrated to one-twentieth its original volume on the water bath. The residue was extracted twice in redistilled 95 per cent alcohol and finally concentrated to dryness *in vacuo*. The final residue was taken up in 0.1 M phosphate solution (pH 7.5) to one-tenth the volume of the original meat infusion.

All of these substances were sterilized by boiling for 20 minutes.

The results of these experiments were quite similar to those obtained in experiments of the same nature with Pneumococcus (2, 15, 18, 19). The washed anaerobic cells by themselves exhibit practically no reducing or oxygen-consuming powers, although the washed cells of the anaerobic bacilli are not so completely devoid of residual reducing power as are washed pneumococci.[3] When mixed with glucose, meat infusion, or the alcohol-soluble substances from potato or muscle juices, the washed bacteria rapidly reduce both methylene blue and methemoglobin. The constituents of the bacterial cells which function in the reducing actions are thermolabile, as mixtures of the heated washed cells and meat infusion (or other "complementary" substances) exhibit no greater reducing action than do the "complementary" substances themselves.

Approximately identical conditions seem to be required for the consumption of molecular oxygen by the washed anaerobic cells as for the establishment of reducing conditions. Oxyhemoglobin is deoxygenated with rapidity only by those mixtures which actively reduce methemoglobin and methylene blue.

Influence of Exposure of Air upon the Subsequent Reducing Activity of Cultures of Anaerobic Bacteria.

From the early literature (21–24) it is evident that both the bacterial cell and the culture fluid participate in the total reducing activity of bacterial cultures. In the case of pneumococci, the reducing activity is almost entirely dependent upon intracellular systems, and the soluble products of growth which appear in the culture fluid add little or nothing to the total reducing power of the whole broth culture. On the other hand, in the case of anaerobic bacilli, while the major portion of the total reducing power of a broth culture is derived from systems which are resident within the cell, these organisms give rise during growth to substances which themselves contribute additional reducing action. In this instance, the culture fluids entirely free from bacterial cells exhibit a certain degree of reducing power which, however, is but a small part of that manifested by the whole culture. The reducing agents in cell-free filtrates or in the supernatant of anaerobic cultures are comparatively thermostable, resist-

[3] A slight reducing action, however, still persists in the case of washed anaerobic cells, as some reduction may be detected after a week's incubation. The anaerobes apparently are less easily washed free of all reducing substances than is the case with Pneumococcus.

ing boiling for 30 minutes. On the other hand, the reducing agents which are intracellular are easily inactivated by heat and appear to be similar in nature to the thermolabile cellular constituent which has been described in sterile extracts of pneumococci.

The reducing power of broth cultures of anaerobic bacilli suffers a rapid loss upon exposure to air. The loss in reducing power following aeration may be attributed to the inactivation of cellular substances. These reactions would seem to be similar to those already described in the case of sterile pneumococcus extracts (15, 18, 19) of which it has been shown that the loss in reducing power is due to the inactivation of the thermolabile cellular constituent of the reducing system (25). When these thermolabile cellular substances are inactivated the bacterial cell is unable apparently to maintain the reducing conditions which depend upon the activity of cellular constituents of this nature.

Oxidation of Hemoglobin and Reduction of Methemoglobin by Sterile Plant Tissue.

That plant tissue in the presence of molecular oxygen forms oxidizing agents of peroxide nature is one of the oldest observations in plant physiology. In previous experiments living pneumococci, sterile extracts of pneumococci, and anaerobic bacilli have been found under appropriate conditions of oxygen tension to exhibit properties of oxidation and reduction. These organisms have been found capable of converting hemoglobin to methemoglobin in the presence of molecular oxygen. The active oxidizing agents concerned in these reactions appear to be similar in nature to the substances formed when plant tissue is exposed to air. Similarly the reducing powers of the bacterial cells are paralleled by analogous reactions in plant tissues. Although possessing these properties in common, the plant tissue differs from the particular group of microorganisms studied in that it is generously endowed with catalase and peroxidase, enzymes which destroy peroxides.

Because of these relationships the oxidizing-reducing action of sterile plant tissue upon hemoglobin and methemoglobin has been tested.

Sterile Plant Tissue.—As examples of the action of plant tissue, potato has been selected since the peroxides formed by this tissue are readily demonstrable. Sterile pieces of potato were obtained by the aseptic procedure used in this laboratory in the preparation of "potato broth" for microbial culture media (26).

Immediately after its excision, the sterile potato tissue was dropped into tubes containing sterile solutions of oxyhemoglobin and of methemoglobin. The tubes of the methemoglobin reduction series were

TABLE IV.

Oxidation of Hemoglobin and Reduction of Methemoglobin by Sterile Plant Tissue.

Oxidation of Hemoglobin.

HbO_2 + potato tissue.	Ferrous hemoglobin (HbO_2 + Hb).	Hb oxidized.
	mM	mM
Original	3.44	—
After 12 hrs. at 25°C	2.59	0.85

Reduction of Methemoglobin.

Methemoglobin* + potato tissue.	Total hemoglobin (HbO_2 + Hb + MetHb).	O_2 content (HbO_2 + O_2).	Ferrous hemoglobin (HbO_2 + Hb).	Ferric hemoglobin (MetHb).†	MetHb reduced.
	mM	mM	mM	mM	mM
Original	3.35	0.7	0.40	2.95	—
After 24 hrs. at 25°C	3.35	0.0	1.09	2.26	0.69
" 48 " " 25° "	3.35	0.0	1.68	1.67	1.28

* A mixture of MetHb, HbO_2, and Hb.

† Total hemoglobin minus ferrous hemoglobin.

sealed with sterile vaseline. Both the hemoglobin oxidation (unsealed) and the methemoglobin reduction (sealed) series of tubes were incubated at 25°C. Analyses of oxygen content and hemoglobin determinations were made at the periods shown in the protocol. The sterility of all test mixtures was proved by subcultures made at the time of the analyses.

Typical examples of the results are presented in Table IV.

The data presented in Table IV illustrate the action of sterile plant tissue upon hemoglobin and methemoglobin. Like pneumococci

and certain anaerobic bacilli, plant tissue can oxidize hemoglobin or reduce methemoglobin, the reaction induced being dependent upon the tension of molecular oxygen. As previously illustrated in the case of anaerobic bacilli and sterile animal tissue, the potato tissue can establish its own reducing conditions in a sealed system initially containing a considerable amount of molecular oxygen. In the absence of molecular oxygen, the potato tissue reduces methemoglobin to hemoglobin. In the presence of molecular oxygen, hemoglobin is oxidized to methemoglobin. It is quite probable that the active oxidizing agents involved in methemoglobin formation are the peroxides formed by the union of molecular oxygen with autoxidizable constituents of the potato, such as are the lipoidal substances isolated by Gallagher (27). The oxidation of hemoglobin to methemoglobin by the potato, thus, is a reaction quite similar to the oxidation of guaiac to its blue oxidation products. With pneumococci, both hemoglobin oxidation and methemoglobin reduction involve the participation of a thermolabile cellular constituent. In the case of potato, however, both of these reactions can be induced by thermostable alcohol-soluble substances, quite independent of ferment participation.

DISCUSSION.

In a study of the oxidation-reduction of blood pigments by anaerobic bacilli, certain resemblances are revealed between the action of these organisms and that of pneumococci. Depending upon the tension of molecular oxygen, both pneumococci and the anaerobic bacilli studied oxidize hemoglobin or reduce methemoglobin. The oxidizing activity of pneumococci in the presence of air is much more marked than is their reducing activity in the absence of air. On the other hand, the reducing activity is much more pronounced in the case of the anaerobic bacilli. These differences are well exemplified by the action of these two groups of microorganisms upon the blood pigments. The readiness with which pneumococci oxidize hemoglobin and the facility with which anaerobes reduce methemoglobin are illustrations of these differences.

Principles similar to those previously described for pneumococci seem to be involved in the operation of some at least of the oxidation-

reduction systems of anaerobic bacilli. The washed cells by themselves can neither consume molecular oxygen nor establish strong reducing conditions; the rapid reduction of methemoglobin and of methylene blue occur only when certain substances such as glucose, meat infusion, and so forth, are added to the washed cells.[3] The optimum oxygen tension for the oxidation of hemoglobin by anaerobic bacilli agrees with that previously established for pneumococci (15). The optimum conditions are provided when the oxygen tension is sufficient for the formation of an active concentration of oxidizing agents without preventing the necessary oxygen dissociation of the oxyhemoglobin. In the absence of oxygen, anaerobes do not oxidize hemoglobin to methemoglobin. Indeed, experiments to be reported later show that if hemoglobin is stored in the presence of these bacteria, in sealed containers, the reducing conditions provided greatly inhibit or wholly prevent the "spontaneous" formation of methemoglobin which ordinarily occurs in sterile blood or oxyhemoglobin solutions.

While it is desired not to project too far the experimental facts observed, the formation of oxidizing agents by broth suspensions of anaerobic bacteria when saturated with air, presents certain immediate and interesting suggestions. It is possible that the same oxidizing agents which oxidize hemoglobin are involved in the injurious action of air upon the more strict anaerobic bacteria. Since the hemotoxin, enzymes, and other cellular constituents of pneumococci are inactivated by oxidizing agents of this nature, the injurious action of air upon anaerobic bacteria may involve a similar inactivation of important cellular constituents. Anaerobes are apparently devoid of catalase and Avery and Morgan (6) and McLeod and Gordon (7) have suggested that the failure of anaerobes to grow in the presence of air is due to the injurious action of peroxides formed by the union of molecular oxygen with autoxidizable cell constituents. In spite of the absence of catalase[1] in anaerobic bacteria, attempts to demonstrate the formation of peroxides by anaerobes have been unsuccessful (McLeod and Gordon (7), Callow (8)). However, peroxides are rapidly destroyed by reducing agents and moreover the detection of peroxide when added to broth itself is much more difficult and the test is much less delicate than in dilute, aqueous solutions.

The experiments presented also illustrate "the native ability of anaerobes to reduce their environment, and thus create their own anaerobiosis if not overtaxed" (Clark (5)). If large numbers of actively growing cells are removed from an anaerobic culture at the height of its growth activity, they consume molecular oxygen with astonishing avidity. If the system be sealed to prevent continued access of air, the anaerobes consume every detectable trace of molecular oxygen even in the face of a considerable reserve provided by the dissociation of molecular oxygen from oxyhemoglobin. The establishment of reducing conditions in such systems is of some theoretical interest. Clark (14, 28) has pointed out that it is theoretically impossible for significant amounts of molecular oxygen to exist at the reduction potentials manifested in cultures of anaerobic bacteria, *provided* that molecular oxygen is actually in equilibrium with the system. Our data show that if any molecular oxygen persists in the above systems of initially high oxygen content, it is in amounts impossible to detect by gasometric methods.

The inherent reducing power of anaerobic bacteria which is illustrated by their action upon blood pigments probably also offers considerable protection to the anaerobic cell when exposed to molecular oxygen. Since this function is resident in the cell it is evident that when exposed to air large inocula of young anaerobes can establish reducing conditions and survive when smaller numbers of the same cells would be "overtaxed," or in fact killed.

The tissue of potato, like Pneumococcus, forms peroxides upon exposure to air, but the vegetable tissue, unlike pneumococci and anaerobic bacilli, is rich in catalase. The potato tissue, like the two groups of bacteria, both oxidizes and reduces the blood pigments. In the absence of molecular oxygen the sterile vegetable tissue converts methemoglobin to hemoglobin and in the presence of air oxidizes hemoglobin to methemoglobin.

SUMMARY.

The oxidation-reduction systems of anaerobic bacteria manifest their action upon the blood pigments by marked deoxygenation of oxyhemoglobin, prompt reduction of methemoglobin to hemoglobin, and to a less marked degree oxidation of hemoglobin to methemoglobin.

The action of sterile plant tissue upon oxyhemoglobin, hemoglobin, and methemoglobin has been studied. The oxidations and reductions of the blood pigments which are induced by sterile plant tissue are similar to the reactions which are brought about by pneumococci and by anaerobic bacteria.

BIBLIOGRAPHY.

1. Conant, J. B., *J. Biol. Chem.*, 1923, lvii, 401.
2. Neill, J. M., *J. Exp. Med.*, 1925, xli, 299.
3. Avery, O. T., and Neill, J. M., *J. Exp. Med.*, 1924, xxxix, 745.
4. Neill, J. M., and Avery, O. T., *J. Exp. Med.*, 1924, xl, 405.
5. Clark, W. M., *J. Washington Acad. Sc.*, 1924, xiv, 123.
6. Avery, O. T., and Morgan, H. J., *Proc. Soc. Exp. Biol. and Med.*, 1923–24, xxi, 59.
7. McLeod, J. W., and Gordon, J., *J. Path. and Bact.*, 1923, xxvi, 332.
8. Callow, A. B., *J. Path. and Bact.*, 1923, xxvi, 320.
9. Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, 1924, lxi, 523.
10. Harington, C. R., and Van Slyke, D. D., *J. Biol. Chem.*, 1924, lxi, 575.
11. Stadie, W. C., *J. Biol. Chem.*, 1920, xli, 237.
12. Gillespie, L. J., *Soil Sc.*, 1920, ix, 199.
13. Clark, W. M., *Pub. Health Rep., U. S. P. H.*, 1923, xxxviii, 443.
14. Clark, W. M., Address before The Chemists' Club, New York, 1924.
15. Neill, J. M., and Avery, O. T., *J. Exp. Med.*, 1924, xxxix, 757.
16. Austin, J. H., Cullen, G. E., Hastings, A. B., McLean, F. C., Peters, J. P., and Van Slyke, D. D., *J. Biol. Chem.*, 1922, liv, 121.
17. Cole, R., *J. Exp. Med.*, 1914, xx, 363.
18. Avery, O. T., and Neill, J. M., *J. Exp. Med.*, 1924, xxxix, 357.
19. Avery, O. T., and Neill, J. M., *J. Exp. Med.*, 1924, xxxix, 543.
20. Harden, A., and Zilva, S. S., *Biochem. J.*, 1915, ix, 379.
21. Labbe, M., *Compt. rend. Soc. biol.*, 1903, lv, 201.
22. Müller, F., *Centr. Bakt., 1. Abt.*, 1899, xxvi, 51, 801.
23. Carapelle, E., *Centr. Bakt., 1. Abt., Orig.*, 1908, xlvii, 545.
24. von Liebermann, L., Jr., *Centr. Bakt., 1. Abt., Orig.*, 1909, li, 440.
25. Neill, J. M., and Avery, O. T., *J. Exp. Med.*, 1925, xli, 285.
26. Thjötta, T., and Avery, O. T., *J. Exp. Med.*, 1921, xxxiv, 97.
27. Gallagher, P. H., *Biochem. J.*, 1923, xvii, 515.
28. Clark, W. M., *Abstr. Bact.*, 1920, iv, 2.

STUDIES ON THE OXIDATION-REDUCTION OF HEMOGLOBIN AND METHEMOGLOBIN.

III. The Formation of Methemoglobin during the Oxidation of Autoxidizable Substances.

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INTRODUCTION.

The present paper reports a study of the oxidation of hemoglobin to methemoglobin by agents formed by the union of molecular oxygen with different autoxidizable[1] substances. The mechanism involved bears obvious relations to other well known examples of the same type of oxidation, such as the oxidation of indigo by benzaldehyde (1), the oxidation of guaiac by plant tissue (2–6), and the oxidation of hemoglobin by Pneumococcus (7, 8). In each of these examples of "oxygen activation" at least two reactions are involved: the formation of an oxidizing agent of peroxide nature, and the subsequent oxidation of a substance itself not reactive with molecular oxygen. The distinctions between these known examples of "oxygen activation" concern the participation of catalysts in the formation of the oxidizing agents, and the participation of catalysts ("activation" of the oxidizing agent) in the subsequent oxidation of the more difficultly oxidized substance.

Hence, in the present study of the oxidation of hemoglobin by autoxidizable substances, the experiments have included measurements both of the consumption of molecular oxygen and of the oxida-

[1] The term "autoxidizable" substance as used in this paper refers to easily oxidized substances which react with molecular oxygen in the absence of thermolabile cellular agents. It is well to point out that hemoglobin has been present in all of the systems studied since the iron of the blood pigments may influence the speed of the oxidations of the "autoxidizable substances."

tion of hemoglobin which occurs during the autoxidations. Experiments were made as well to determine if the presence of thermolabile cellular substances of pneumococci accelerates the oxygen-consuming and hemoglobin-oxidizing activities of the autoxidizable substances. In analyzing the mechanism of the formation of methemoglobin in these reactions, it is well to remember that the hemoglobin molecule itself possesses the property of catalyzing the oxidations induced by certain peroxides.

EXPERIMENTAL.

Methods.

Bacteriological.—The sterile pneumococcus extracts used in this investigation were prepared as described in previous papers (9). All reagents used in the experiments were sterile. The usual aseptic, bacteriological technique was employed throughout all experiments to preclude any possibility of the participation of bacterial action in the reactions which have been ascribed to autoxidation.

Chemical.—The alcoholic extracts of potato juice and meat infusion were prepared as previously described (10). The alcohol was redistilled immediately before using. Unless redistilled, the alcohol yielded a residue upon evaporation, which when dissolved in phosphate solution oxidized hemoglobin to methemoglobin.

The gasometric measurements of "oxygen contents" and "oxygen capacities" were made with the apparatus and technique described by Van Slyke and Neill (11). All reagents were adjusted to pH 7.5; the pH was maintained approximately constant, as the phosphate concentration of all reaction mixtures was at least 25 mM.

The technique and apparatus described by Robertson and Sia (12) proved useful in maintaining good admixtures of hemoglobin solutions with substances such as turpentine, linseed oil, etc.

The Oxidation of Hemoglobin to Methemoglobin by Oxidizing Agents Formed during the Autoxidation of Turpentine, Cod Liver Oil, and Linseed Oil.

Turpentine, cod liver oil, and linseed oil were chosen as classical examples of liquids containing unsaturated substances which form peroxides when exposed to molecular oxygen (5, 13, 2, 14). *Aerated* samples of these liquids are, in fact, frequently substituted for hydrogen peroxide in testing for the presence of blood or hemoglobin by the peroxidase-guaiac or peroxidase-benzidine test.

The results of a number of experiments showed that if turpentine, cod liver oil, or linseed oil is added to oxyhemoglobin, molecular oxygen is consumed with the formation of oxidizing agents which oxidize hemoglobin to methemoglobin. So far as could be determined the rate of methemoglobin formation by these substances was not accelerated to a significant degree by the thermolabile cellular substances of washed pneumococci. The formation of methemoglobin by turpentine, linseed oil, and cod liver oil in the presence of oxyhemoglobin can be interpreted as reactions analogous to the oxidation of guaiac in peroxidase-guaiac-peroxide reactions.

Oxygen Consumption and Methemoglobin Formation during the Autoxidation of Alcoholic Extracts of Potato Juice.

Gallagher (5) has shown that alcoholic extracts of potato juice contain unsaturated substances which form peroxides when exposed to the air. Although the alcohol-soluble substances from potato are autoxidizable and can form peroxides independent of ferment action, it seemed quite probable that the thermolabile cellular substances of Pneumococcus might accelerate the consumption of molecular oxygen and the formation of oxidizing agents by the potato extracts. The peroxides formed by the potato extracts are probably similar in nature to the oxidizing agents formed in sterile broth extracts of pneumococci and might be expected to oxidize hemoglobin to methemoglobin.

Qualitative experiments revealed that if the alcohol-soluble substances themselves were added to solutions of oxyhemoglobin, methemoglobin was formed. However, if thermolabile cellular substances of washed pneumococci (which by themselves produce no change in oxyhemoglobin solutions) were also present the formation of methemoglobin was greatly accelerated.

In quantitative experiments comparisons were made of the oxygen consumption and the hemoglobin oxidation which is induced by alcoholic extracts of potato juice both in the presence and in the absence of thermolabile constituents of the pneumococcus cell.

Suspensions of washed intact pneumococci, sterile solutions of oxyhemoglobin, phosphate solutions of the alcohol-soluble substances extracted from potato juice, and sterile phosphate solution were used to complete the reaction mixtures. The tubes containing the mixtures indicated in the protocol were then sealed with sterile vaseline,

and incubated in a water bath at 37°C. for 2 hours. At the end of this period, "oxygen content" analyses were made to determine the consumption of molecular oxygen, and "oxygen capacity" analyses, to determine the amount of hemoglobin which had been oxidized to methemoglobin. The presence of methemoglobin was confirmed by spectroscopic observations.

Table I presents the protocol of a typical experiment.

It is evident (Table I) that during the autoxidation of the alcohol-soluble substances of potato juice molecular oxygen is consumed. Presumably during this reaction, oxidizing agents (probably peroxides) are formed which in turn oxidize hemoglobin to methemoglobin.

TABLE I.

Autoxidation of Alcohol-Soluble Substances of Potato Juice, and the Acceleration of the Reactions by Labile Constituents of Washed Pneumococci.

Suspension of washed pneumococci.		Alcohol-soluble substances from potato juice.	HbO_2	PO_4 solution.	"O_2 content" (HbO_2 + O_2).	"O_2 capacity" (HbO_2 + Hb).	Oxygen consumed.	Hb oxidized.
Unheated.	Heated.							
cc.	*cc.*	*cc.*	*cc.*	*cc.*	*mM*	*mM*	*mM*	*mM*
1.5	—	1.5	3.0	—	0.00	1.11	3.95	2.58
—	1.5	1.5	3.0	—	2.48	2.66	1.47	1.03
—	—	1.5	3.0	1.5	2.53	2.64	1.42	1.05
1.5	—	—	3.0	1.5	3.80	3.54	0.15	0.15
—	—	—	3.0	3.0	3.95	3.69	—	—

These reactions can be induced quite independently of any possible participation of cellular substances of ferment nature. However, it is evident as well that in the presence of thermolabile constituents of pneumococcus cells,—which by themselves are quite non-reactive,—the consumption of molecular oxygen and the subsequent oxidation of hemoglobin are greatly accelerated. Results of the same nature were obtained in experiments in which sterile extracts of washed pneumococci were used in the place of suspensions of washed intact cells.

Oxygen Consumption and Methemoglobin Formation during the Autoxidation of the Alcohol-Soluble Substances of Meat Infusion.

Experiments analogous to the preceding ones have been conducted with alcohol-soluble substances extracted from the meat infusion which

is used in the preparation of bacteriological culture media. These substances are autoxidizable and by themselves consume molecular oxygen with the formation of oxidizing agents which oxidize hemoglobin to methemoglobin.

In the presence of the thermolabile intracellular substances of washed pneumococci, both oxygen consumption and hemoglobin oxidation are greatly accelerated. The oxygen consumption and methemoglobin formation during the *autoxidation* of the alcohol-soluble substances of meat infusion are definite but are somewhat less pronounced in the absence of thermolabile cellular substances than those which occur during the autoxidation of the alcohol-soluble substances of potato juice.

Evidently, some at least of the thermostable substances used to "complete" the "incomplete" oxidation-reduction systems in sterile saline extracts of washed pneumococci (7–9) are themselves autoxidizable, but both the consumption of molecular oxygen and the formation of oxidizing agents, which processes proceed slowly in the absence of thermolabile cellular substance, are greatly accelerated by the presence of thermolabile substances derived from the washed pneumococcus cell.

The Oxidation of Hemoglobin by Oxidizing Agents Formed during the Autoxidation of Oleic and Linolic Acids.

Gallagher (5) found that oleic acid formed peroxides when exposed to molecular oxygen. Accordingly, it seemed that, if oleic acid formed oxidizing agents which oxidized guaiac in the presence of plant peroxidase, the same agents under like conditions might also oxidize hemoglobin to methemoglobin. The catalytic function of the plant peroxidase in guaiac oxidation, if necessary in hemoglobin oxidation, might be performed by the iron of the hemoglobin molecule itself. Experiments were made to test these relations.

Samples of pure oleic acid and linolic acid were obtained through the courtesy of Dr. Ida P. Rolf of The Rockefeller Institute. 2 per cent solutions of the two acids were prepared in 0.1 M PO_4 solution. Sterile NaOH was added to give a pH of 7.5.

Dr. Rolf has furnished the following description of the preparation and analyses of the samples of linolic acid, oleic acid, and lecithin, which were used in the experiments.

Linolic Acid.—Kahlbaum's best grade of linolic acid was converted to the methyl ester and fractionally distilled *in vacuo*. The constant boiling fraction was saponified with NaOH and the soap converted to the free acid. The acid had an iodine number (Wij's determination) of 179 (theoretical, 180). The acid, on titration with 0.1 N NaOH showed a molecular weight of 281 (theoretical, 280). After hydrogenation, the saturated acid analyzed as follows: C, 75.93; H, 12.64 (theoretical calculation for $C_{18}H_{36}O_2$: C, 75.98; H, 12.76) and melted at 71°C.

Oleic Acid.—Kahlbaum's oleic acid was converted to the methyl ester and fractionally distilled *in vacuo*. That fraction used after transformation to the acid (as above, for linolic acid) had the following properties: melted at 14°C.; had an iodine value of 80; after hydrogenation, had a melting point of 71°C. and analyzed: C, 75.86; H, 12.57.

Lecithin.—The lecithin was prepared from egg oil through the cadmium chloride salt. It was amino-free and had an iodine number of 35. It analyzed: C, 65.09; H, 10.76; N, 1.98; P, 3.80 (calculated as $C_{43}H_{86}O_9NP$: C, 65.26; H, 10.94; N, 1.97; P, 3.92).

When added to solutions of oxyhemoglobin, the oleate and linolate emulsions formed significant amounts of methemoglobin. Similar tests on a sample of lecithin gave negative results. Tests were also made to determine if the thermolabile cellular substances of Pneumococcus accelerate the hemoglobin oxidation. It was found, however, that the cellular substances are apparently without effect upon the methemoglobin formation which is induced during the autoxidation of oleic and linolic acids.

These results are of some significance. Examples are furnished of the fact that, during the autoxidation of oleic and linolic acids, at least in the presence of the iron of hemoglobin, a second substance (hemoglobin) can be oxidized which by itself is oxidized difficultly if at all by molecular oxygen. Either of these two unsaturated acids plus oxyhemoglobin constitutes, then, a system which oxidizes hemoglobin in a manner analogous to the oxidation of guaiac by the oxidase system of potato tissue. In neither of these two systems, unlike the system by which Pneumococcus oxidizes hemoglobin, is a thermolabile cellular substance essential for the formation of the oxidizing agent.

The Influence of the Tension of Molecular Oxygen upon the Formation of Methemoglobin during the Oxidation of Autoxidizable Substances.

In the oxidation of hemoglobin by pneumococci, the tension of molecular oxygen determines both the formation of the oxidizing agent and the concentration of hemoglobin, the substance actually oxidized to methemoglobin. Experiments to be reported in another paper (15) show that the oxidation of hemoglobin by the autoxidizable substances is influenced by the oxygen tension in the same manner as is the oxidation of hemoglobin by sterile pneumococcus extracts. In the complete absence of molecular oxygen, little or no methemoglobin is formed, presumably owing to the fact that oxidizing agents are not formed. However, at high tensions of molecular oxygen, the formation of methemoglobin is less marked than at tensions which permit the oxygen dissociation of about half of the oxyhemoglobin. These experiments are quoted in this place as further evidence of the validity of considering the oxidations of hemoglobin by pneumococci and by autoxidizable substances to be reactions of the same general type.

There is also an evident analogy between this type of hemoglobin oxidation and the oxidation of hemoglobin by hydroquinone and aminophenol. Huebner (16) found that neither hydroquinone nor aminophenol formed methemoglobin from hemoglobin in the absence of oxygen. When these reduced substances were oxidized, however, the oxidation products rapidly oxidized hemoglobin to methemoglobin. Apparently, as in the case of the autoxidizable substances reported in this paper, hydroquinone and aminophenol unite with molecular oxygen to form oxidizing agents which subsequently oxidize the iron of the hemoglobin molecule.

The alcohol-soluble constituents of potato juice exhibit another relation to oxygen tension which is analogous to that exhibited by pneumococci and by the intact potato tissue. It has previously been shown that Pneumococcus and sterile potato tissue possess both oxidizing and reducing properties and, hence, can either oxidize hemoglobin or reduce methemoglobin, depending upon the tension of molecular oxygen. A mixture of the alcohol-soluble substances from potato exhibits the same relations and oxidizes or reduces the blood pigments, the direction of the reaction being dependent upon the presence or absence of molecular oxygen.

DISCUSSION.

In the experiments reported it was found that hemoglobin may be oxidized to methemoglobin by agents formed during the autoxidation of pure oleic and linolic acids and of substances present in turpentine, cod liver oil, and linseed oil. In these cases, the participation of thermolabile cellular substances is not required, either for the autoxidations or for the oxidation of hemoglobin by the oxidizing agents formed during the autoxidation.

Autoxidizable substances were extracted by alcohol from meat infusion and from potato juice. Mixtures of the alcohol-soluble substances from either the animal or plant juices consumed molecular oxygen with the formation of oxidizing agents capable of oxidizing hemoglobin to methemoglobin. Both oxygen consumption and hemoglobin oxidation by these alcoholic extracts occurred in the absence of the thermolabile substances of Pneumococcus. However, both of these reactions were greatly accelerated in the presence of cellular derivatives of washed pneumococci, which, as previously shown, are by themselves wholly inactive. In a preceding paper (17) it was concluded that these bacterial constituents function as catalysts in the biological oxidations induced by pneumococci. In contrast to the alcoholic extracts of plant and animal tissue the reactions induced by oleic and linolic acids apparently are not accelerated by the addition of the labile bacterial substances.

The oxidations of hemoglobin by pneumococci and by the oxidizing agents formed during the autoxidations studied in this paper, bear certain analogies to the oxidation of guaiac by plant tissue. In both cases, at least two reactions are involved: (1) the formation of an oxidizing agent by the union of molecular oxygen with an autoxidizable or easily oxidized substance; (2) the oxidation of the more difficultly oxidized substance (hemoglobin or guaiac) by the oxidizing agent formed in the first reaction. It is possible that in the formation of methemoglobin the iron of the hemoglobin molecule functions as peroxidase in the "activation" of the oxidizing agent in a manner analogous to the action of the peroxidase of vegetable tissue in the guaiac reaction.

While hemoglobin is oxidized to methemoglobin if oxygen is ad-

mitted to mixtures of the blood pigments and the easily oxidized substances studied in this paper, the reverse reaction is induced if oxygen is excluded from the same mixtures. Thus, the presence or absence of molecular oxygen determines the direction of the reaction induced by these substances in a manner analogous to that previously shown in the case of pneumococci.

SUMMARY.

Hemoglobin is oxidized to methemoglobin by products formed during the oxidation of a number of different substances. With some of these substances, both oxygen consumption and hemoglobin oxidation are accelerated by thermolabile constituents of pneumococci; with others of the easily oxidized substances, neither reaction seems to be influenced by the presence of the bacterial substances.

The same substances which in the presence of air induce the oxidation of hemoglobin bring about the reverse reaction if molecular oxygen is excluded.

BIBLIOGRAPHY.

1. Bach, A., *Compt. rend. Acad.*, 1897, cxxiv, 951.
2. Kastle, J. H., *Bull. Hyg. Lab., U. S. P. H., No. 59*, 1910.
3. Dakin, H. D., Oxidations and reductions in the animal body, London, New York, Toronto, Bombay, Calcutta, and Madras, 1922, 4–15. Engler, C., *Ber. chem. Ges.*, 1900, xxxiii, 1097. Engler, C., and Wild, W., *Ber. chem. Ges.*, 1897, xxx, 1669. Engler, C., and Herzog, R. O., *Z. physiol. Chem.*, 1909, lix, 327. Battelli, F., and Stern, L., *Ergebn. Physiol.*, 1912, xii, 96; *Arch. internat. physiol.*, 1921, xviii, 403. Zelinsky, N. D., and Borissow, P. P., *Ber. chem. Ges.*, 1923, lvi, 396.
4. Bach, A., and Chodat, R., *Ber. chem. Ges.*, 1902, xxxv, 2466; 1903, xxxvi, 606, 1756.
5. Gallagher, P. H., *Biochem. J.*, 1923, xvii, 515.
6. Schönbein, *Ann. Physik u. Chem.*, 1848, lxxv, 357.
7. Neill, J. M., and Avery, O. T., *J. Exp. Med.*, 1924, xxxix, 757.
8. Neill, J. M., *J. Exp. Med.*, 1925, xli, 299.
9. Avery, O. T., and Neill, J. M., *J. Exp. Med.*, 1924, xxxix, 357.
10. Neill, J. M., *J. Exp. Med.*, 1925, xli, 535.
11. Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, 1924, lxi, 523.
12. Robertson, O. H., and Sia, R. H. P., *J. Exp. Med.*, 1924, xxxix, 219.
13. Ramage, A. S., U. S. Patent, May 26, 1914; *Chem. Abstr.*, 1914, viii, 2603.

14. Lewkowitsch, J., Chemical technology and analysis of oils, fats, and waxes, London, 5th edition, 1915, iii, 169. Rideal, E. K., and Taylor, H. S., Catalysis in theory and practice, London, 1919, 138–155.
15. Neill, J. M., and Hastings, A. B., *J. Biol. Chem.*, 1925, lxiii, 479.
16. Huebner, W., *Arch. exp. Path. u. Pharmakol.*, 1913, lxxii, 241.
17. Neill, J. M., and Avery, O. T., *J. Exp. Med.*, 1925, xli, 285.

STUDIES ON THE OXIDATION-REDUCTION OF HEMOGLOBIN AND METHEMOGLOBIN.

IV. The Inhibition of "Spontaneous" Methemoglobin Formation.

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The apparently spontaneous conversion of hemoglobin to methemoglobin is an occurrence that has been recognized ever since the discovery of the latter blood pigment (Hoppe-Seyler, 1864). Although certain bacteria can oxidize hemoglobin to methemoglobin, the alteration of hemoglobin observed by the chemist is probably rarely due to bacterial action. Indeed, this conversion can occur in the complete absence of bacteria, taking place in blood drawn and held under aseptic conditions and in solutions of crystalline oxyhemoglobin sterilized by Berkefeld filtration.

The so called "spontaneous" methemoglobin formation in drawn blood and in hemoglobin solutions seems to be the result of autoxidation of the hemoglobin itself or of other blood constituents. Some evidence indicative of the conditions influencing the "spontaneous" alteration of hemoglobin is presented in this paper. The investigation has consisted in large part of attempts to determine whether reducing conditions established by biological agents inhibit the transformation of hemoglobin to methemoglobin.

The "spontaneous" conversion of hemoglobin was observed by the earlier workers on blood pigment and led directly to the discovery and study of methemoglobin (early references cited in a preceding paper (1)). The temperature of storage apparently affects only the rate of the reaction. When solutions of oxyhemoglobin are dried under usual conditions, the oxygen-carrying power of the hemoglobin is greatly diminished or entirely lost. Bohr (2) dried crystals of oxyhemoglobin under different conditions. Solutions of the dried crystals possessed different oxygen-combining power, and he concluded that this was the result of the presence of different modifications of oxyhemoglobin. He reported that solutions

of his "beta-oxyhemoglobin" were spectroscopically identical with his "alpha-oxyhemoglobin," although the oxygen-carrying value of the "beta-oxyhemoglobin" was considerably lower. Hüfner (3), however, concluded that Bohr's solutions which exhibited different oxygen capacities consisted of different mixtures of intact and of partially destroyed hemoglobin. It is evident that Bohr's dried hemoglobin had undergone considerable alteration, since the solutions of "beta-oxyhemoglobin" had to be clarified by prolonged centrifugation. The insoluble material may have been globin which Preyer (1868) (4) had previously found in solutions of methemoglobin crystals prepared by drying hemoglobin under conditions similar to those used by Bohr in the preparation of "beta-oxyhemoglobin." The preparation of pure crystalline oxyhemoglobin is complicated not only by the fact that the use of reagents which contain oxidizing agents, such as ethylhydrogen peroxide in the ether, among others, must be avoided, but also by the fact that it seems to be exceedingly difficult to free the hemoglobin from traces of autoxidizable blood constituents. Solutions of apparently pure oxyhemoglobin crystals frequently include substances which consume molecular oxygen and form carbon dioxide.[1]

EXPERIMENTAL.

Methods.—All reagents used in the experiments were sterile. The oxyhemoglobin solutions were prepared without crystallization from the well washed cells of sterile, defibrinated horse and rabbit blood. The cells were laked in sterile distilled water and were frozen and thawed several times. Sterile phosphate solution was added in amount sufficient to give a final phosphate concentration of 20 mM. These solutions were then centrifuged.

The sterile, laked blood was obtained by freezing and thawing defibrinated horse blood; the laked blood was centrifuged to remove cell detritus.

The measurements of "oxygen content" and the determination of hemoglobin were made by the gasometric methods described by Van Slyke and Neill (7).

The anaerobic bacilli used were strains of *B. histolyticus* and *B. aerofœtidis*.

I. Inhibition of the "Spontaneous" Formation of Methemoglobin in Blood at Ordinary Temperatures.

Preceding papers (1, 8) have shown that bacteria rapidly establish reducing conditions in blood or hemoglobin solutions if free access of air is prevented. Experiments were conducted to determine if the reducing conditions established by this biological method would prevent the "spontaneous" formation of methemoglobin which occurs in sterile blood. Reducing conditions were obtained in sterile laked

[1] Unpublished observations.

blood by adding suspensions of anaerobic bacilli (*Bacillus histolyticus*, *Bacillus aerofœtidis*) and of the aerobic organism, *Bacillus coli*, which, among the aerobic bacteria, possesses particularly strong reducing powers. As previously shown, the bacteria rapidly consume all of the dissolved oxygen and also that dissociated from oxyhemoglobin. The hemoglobin reduced in this manner, and the same hemoglobin solution to which no bacteria had been added, were then stored under the same conditions and subsequently tested for "oxygen content" and "oxygen capacity."

Experiments to be reported in another paper show that "spontaneous" methemoglobin formation in sterile blood proceeds most rapidly at a diminished tension of molecular oxygen. In recognition of this fact, some of the tubes of sterile blood were sealed with vaseline to prevent further entrance of oxygen. In these closed systems, a somewhat reduced oxygen tension was soon provided by the oxygen consumption of the sterile, laked blood.

The hemoglobin was completely oxygenated in all the tubes at the beginning of the experiments. (1) In the series containing the bacteria, the tubes were sealed after the addition of the bacteria. The bacterial cells rapidly established their own reducing conditions even in the face of the reserve supply of molecular oxygen furnished by the dissociation of oxyhemoglobin. (2) In the unsealed tubes, the oxygen tension of the control samples was probably little less than that of the atmosphere, although the tubes were not agitated. (3) In the sterile blood in the sealed tubes, the oxygen tension was considerably diminished after several days, due to oxygen consumption by the blood itself.

After different periods of storage, "oxygen content" and "oxygen capacity" analyses were made. The protocols of typical experiments are given in Table I.

The following relations are evidenced from the data presented in Table I. (1) Little or no methemoglobin can accumulate in blood which is held under the strong reducing conditions established by bacterial cells in a closed system. (2) If sterile blood is held in a closed system under slightly diminished oxygen tension more methemoglobin is formed than is formed in an open system in which most of the hemoglobin remains as oxyhemoglobin.

As shown in the data presented, a slight decrease in "oxygen capacity" occurred in the blood containing the anaerobic bacteria after 5 to 14 days storage. Since no methemoglobin was ever detected spectroscopically in the samples reduced by the bacterial cells, it is doubtful if the slight but definite loss in "oxygen capacity" was due to methemoglobin formation. The most probable explanation of this

loss in "oxygen capacity" is that some of the hemoglobin had combined with hydrogen sulfide or other gaseous products of bacterial putrefaction, and that these gases were not completely displaced by oxygen when the samples were "saturated" with air at the time of the analyses. "Total pigment" measurements by the cyanmethemoglobin method showed that the loss in "oxygen capacity" could not be explained by the proteolytic cleavage of the hemoglobin. The fact that little or no hemoglobin was hydrolyzed in the presence of large numbers of proteolytic

TABLE I.

Inhibition of "Spontaneous" Methemoglobin Formation at Room Temperature by the Reducing Conditions Established by Bacterial Cells.

	"O_2 content" (HbO_2+O_2).		"O_2 capacity" (HbO_2+Hb).			Hemoglobin destroyed.		MetHb bands detected.
	Original.	After 5 days at 25°C.	Original.	After 5 days at 25°C.	After 13 days at 25°C.	After 5 days at 25°C.		
	mM	mM	mM	mM	mM	*per cent*	*per cent*	
Series I.								
Sterile, laked blood in open tube...	7.79		7.50	6.60		12		+
" " " " sealed tube..	7.79	4.78	7.50	6.01		20		+
Blood in sealed tube, reduced by anaerobic bacilli.....	6.55	0.00	6.26*	5.98		5		−
				7 days at 25°C.		7 days at 25°C.	13 days at 25°C.	
Series II.								
Sterile, laked blood in open tube...			8.70	8.02	6.76	8	22	+
" " " " sealed tube..			8.70	7.64	6.12	12	29	+
Blood in sealed tube, reduced by anaerobic bacilli.....			6.54*	6.45	6.18	1	5	−

* Calculated from dilution of the blood by the measured amount of bacterial suspension added.

bacteria shows that hemoglobin as a protein offers considerable resistance to some at least of the usual protein-hydrolyzing enzymes.

The prevention of the accumulation of methemoglobin in the above experiments is probably due to an actual inhibition of the oxidation of the hemoglobin rather than to the simultaneous reduction of methemoglobin. Later experiments show the reducing conditions established by bacterial cells can prevent subsequent methemoglobin formation at temperatures at which the bacteria are actually killed.

Experiments of the same nature as the preceding ones show that the alcohol-soluble constituents of sterile meat infusion can, like bacterial cells, establish reducing conditions under which the "spontaneous" formation of methemoglobin is inhibited or entirely prevented. If dilute solutions of oxyhemoglobin are placed in sealed tubes, methemoglobin is formed after several days storage at room temperature or at 37°C. However, if a small amount of the alcohol-soluble substances from meat infusion be added to the oxyhemoglobin, the oxygen is consumed and the deoxygenated hemoglobin can be stored for at least 9 months without the formation of a trace of methemoglobin. Dilute solutions of hemoglobin maintained in the reduced state by the action of sterile animal tissues have also been stored for months in sealed tubes without detectable methemoglobin formation. Although in the absence of air these substances establish reducing conditions under which the "spontaneous" formation of methemoglobin is inhibited, the same substances if air is present form oxidizing agents which rapidly oxidize hemoglobin to methemoglobin (9).

II. Inhibition of the "Spontaneous" Formation of Methemoglobin Which Occurs at High Temperatures.

In the early literature, reports are made that methemoglobin is formed rapidly whenever oxyhemoglobin is exposed to high temperatures. Experiments were made to determine the effect of reducing conditions upon the stability of hemoglobin exposed to temperatures of 55°C.

The formation of methemoglobin when blood or solutions of hemoglobin are exposed to high temperatures was observed by the early workers on blood pigments. Hoppe-Seyler (10), however, reports that if oxyhemoglobin crystals are completely free from water, they may be heated to 100°C. without destruction of the hemoglobin. Dittrich (11) pumped off all of the oxygen from solutions of hemoglobin and sealed the glass container and then exposed this oxygen-free hemoglobin to a temperature of 55°C. While methemoglobin was formed rapidly in control tubes, in the presence of oxygen, Dittrich found that no methemoglobin was formed in the oxygen-free hemoglobin even after 2½ days exposure to 55°C. These results seemed of sufficient importance in connection with the present study to justify an attempt to confirm them by the more accurate methods of hemoglobin determination now at hand.

In the following experiments, suspensions of anaerobic bacilli were added to sterile solutions of oxyhemoglobin. The tubes were sealed and incubated at 37°C. for 3 hours, during which time the bacteria consumed all the oxygen and provided

TABLE II.

Stability of Hemoglobin at 55°C. in the Presence and in the Absence of Molecular Oxygen.

	O_2 content at beginning of heating.	Original HbO_2+Hb.	Heated at 55°C. for.	HbO_2+Hb after exposure to 55°C.	Hb destroyed.		Spectroscopic observation.		
							MetHb	Hematin.	HbO_2 or Hb.
	mm	mm	hrs.	mm	mm	per cent			
Series I.									
5 cc. rabbit HbO_2 + 1.0 cc. PO_4 solution	2.10	1.84	3	1.25	0.6	30	+	*	+
5 cc. rabbit HbO_2 + 1.0 cc. broth suspension of anaerobic bacteria	0.00	1.84	3	1.88	0.0	0.0	−	*	+
Series III.									
5 cc. horse HbO_2 + 1.5 cc. PO_4 solution	3.99	3.73	4	*	*	*	+	±	+
5 cc. horse HbO_2 + 1.5 cc. PO_4 solution	3.99	3.73	24	0.00	3.73	100	−	+	−
5 cc. horse HbO_2 + 1.5 cc. broth suspension of anaerobic bacteria	0.00	3.73	24	3.55	0.18	5	−	−	+
5 cc. horse HbO_2 + 1.5 cc. broth suspension of anaerobic bacteria	0.00	3.73	72	3.60	0.13	4	−	−	+
Series V.									
4 cc. rabbit HbO_2 + 1 cc. PO_4 solution	4.50	4.24	24	0.10	4.14	98	−	+	±
4 cc. rabbit HbO_2 + 1 cc. broth suspension of anaerobic bacteria	0.00	4.24	240	3.78	0.46	11	−	−	+

* Not determined.

a reducing system in which all of the blood pigment was in the form of reduced or deoxygenated hemoglobin.

Another series of unsealed tubes containing the same sterile oxyhemoglobin

solution was prepared for use as controls. Both series of tubes, the sealed tubes of reduced hemoglobin and the open tubes of oxyhemoglobin, were then placed in a water bath at 55°C. After different periods of exposure to this temperature, spectroscopic observations and gasometric analyses were made to determine the alteration of the blood pigments. Typical protocols are given in Table II.

As shown by the results of these experiments (Table II) the presence of molecular oxygen markedly affects the degree of stability of hemoglobin at 55°C. Whereas if oxygen is present the hemoglobin is rapidly destroyed to methemoglobin and finally to globin and hematin, the reduced hemoglobin may be exposed to 55°C. for periods as long as 10 days without loss of over 10 per cent in the original "oxygen capacity." Moreover, in spite of the small but significant losses in "oxygen capacity" which occurred it was never possible to detect the presence of methemoglobin in the oxygen-free series. After several days exposure to 55°C., some precipitation of the reduced hemoglobin occurred. This precipitate, however, redissolved when cooled, and despite this physical change was still capable of combining with oxygen.

These experiments confirm Dittrich's early work, since the amount of hemoglobin destroyed in the reduced series after 2 or 3 days heating could be detected only by refined gasometric methods. Dittrich interpreted his results as evidence that oxyhemoglobin is an intermediate product in the formation of methemoglobin from hemoglobin (*i.e.*, Hb→HbO_2→MetHb). This interpretation is contrary to the relations established by Conant (12) and is not in keeping with the principles of the methemoglobin formation obtaining in the biological oxidations reported in previous papers (1, 8, 9, 13). It is more probable that in the reducing conditions established by the bacteria in the above experiments previous to the heating treatment, all of the oxygen was removed and no oxidizing agents could be formed in the system to oxidize the hemoglobin. The reductions were induced, to begin with, by living bacteria, and once established were maintained during the subsequent exposure at 55°C. although continued exposure to this temperature resulted in the death of the bacteria.

On the other hand the results of these experiments indicate that in the presence of oxygen the formation of methemoglobin at 55°C. represents an acceleration of the same oxidation reaction which is

responsible for the "spontaneous" formation of methemoglobin observed at lower temperatures. The temperature of 55°C. is especially suitable for the illustration of this relation, as at slightly higher temperatures the reduced hemoglobin itself is destroyed, apparently without passing through the stage of methemoglobin.

The results of Table II are also of interest from the standpoint of the relative stability of reduced hemoglobin and of methemoglobin. The methemoglobin which is formed in the hemoglobin solutions heated in the presence of oxygen is rapidly destroyed, in part at least, to globin and hematin, while the reduced hemoglobin proves relatively stable at 55°C. It has also been observed that sterile solutions of methemoglobin are less stable at ordinary temperatures than solutions of hemoglobin or oxyhemoglobin. Apparently, the most usual course of the destruction of hemoglobin is by way of methemoglobin (*i.e.* $HbO_2 \rightarrow Hb \rightarrow MetHb \rightarrow$ globin and hematin), the formation of methemoglobin being an intermediate stage in the normal destruction of blood pigment.

DISCUSSION.

Evidence has been reported in the preceding experiments that the "spontaneous" formation of methemoglobin which occurs in sterile drawn blood or in sterile hemoglobin solutions can be prevented by maintaining the hemoglobin in a system rendered oxygen-free by biological reducing agents. This evidence in conjunction with earlier reports in the literature offers certain suggestions concerning the mechanism of the reaction usually involved in such methemoglobin formation. Since methemoglobin is now known to be the oxidation product of hemoglobin, it would seem certain that its "spontaneous" formation is an oxidation. The formation of methemoglobin through the influence of certain light waves may represent simply an increased velocity of the reaction which is responsible for "spontaneous" methemoglobin formation in the dark, as in both cases the presence of oxygen is required. The degree of aeration apparently affects the degree of alteration of hemoglobin during the drying of crystals (2, 3). Moreover, as shown in the present paper, the presence of oxygen also seems necessary for the "spontaneous" formation of methemoglobin in blood or in hemoglobin solutions, either at ordinary temperatures or at 55°C.

The actual oxidizing agents involved in "spontaneous" methemoglobin formation would seem to be either "activated" oxygen, possibly of peroxide nature, or molecular oxygen itself. Ozone, an oxidizing agent similar to those involved in so called oxygen activations, oxidizes hemoglobin to methemoglobin (14, 10). "Spontaneous" methemoglobin formation may be due to the oxidizing agents formed from unsaturated substances in blood. The fact that light waves catalyze the methemoglobin-forming reaction (5) would be explained if unsaturated substances are involved, as light is a classical "activator" or "sensitizer" in the formation of peroxides from unsaturated compounds. In considering the importance of these substances in "spontaneous" methemoglobin formation, it is impossible to ignore the well known peroxide-activating property of the hemoglobin molecule itself.

However, it is possible that "spontaneous" formation of methemoglobin is due to an autoxidation of the hemoglobin itself; *i.e.*, to a slow oxidation by molecular oxygen of hemoglobin in the complete absence of foreign substances. Such a reaction might well be accelerated by temperature or by light. Ferrous salts of the highest purity are frequently autoxidizable and are converted to ferric salts in solutions exposed to the air. A similar reaction may be induced in solutions of the complex ferrous salt which Conant has proposed to represent the hemoglobin molecule.

All of the examples of methemoglobin formation which had been reported in this and the preceding papers represent oxidations of the same general type. The mechanisms involved may be represented graphically as follows:

1. Methemoglobin formation by pneumococci.

$$(a)\begin{cases} \overbrace{C}^{\text{(thermolabile, cellular substance)}} + \overbrace{RH}^{\text{(thermostable, easily oxidized substance)}} + O_2 \rightarrow \overbrace{ROOH}^{\text{(oxidizing agent)}} \\ \text{or} \\ C + 2\,RH + O_2 \rightarrow 2\,R + H_2O_2 \end{cases}$$

$$(b) \qquad ROOH \text{ (or } H_2O_2) + Hb \rightarrow MetHb$$

2. Methemoglobin formation during the autoxidation of other substances.

$$(a)\begin{cases} RH + O_2 \rightarrow ROOH \\ 2\,RH + O_2 \rightarrow 2\,R + H_2O_2 \end{cases}$$

$$(b)\quad ROOH\ (\text{or } H_2O_2) + Hb \rightarrow MetHb$$

As suggested above, the "spontaneous" formation of methemoglobin in pure hemoglobin solutions may involve a slow autoxidation of the hemoglobin by molecular oxygen itself, in which process the iron of the hemoglobin may serve as a catalytic agent.

3. Methemoglobin formation in pure hemoglobin solutions.

$$Hb + x(O_2) \rightarrow MetHb$$

SUMMARY.

It is well known that methemoglobin is gradually formed in sterile blood or hemoglobin solutions which are stored under the usual conditions. If the hemoglobin is reduced by biological agents and stored in the absence of air, the formation of methemoglobin is inhibited, or wholly prevented. The reasons for this are discussed.

BIBLIOGRAPHY.

1. Neill, J. M., *J. Exp. Med.*, 1925, xli, 299.
2. Bohr, C., *Centr. Physiol.*, 1890–91, iv, 249.
3. Hüfner, G., *Arch. Anat. u. Physiol., Physiol. Abt.*, 1894, 130.
4. Preyer, W., *Arch. ges. Physiol.*, 1868, i, 395.
5. Hasselbalch, K. A., *Biochem. Z.*, 1909, xix, 435.
6. Gröber, A., *Arch. exp. Path. u. Pharmakol.*, 1908, lviii, 343.
7. Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, 1924, lxi, 523.
8. Neill, J. M., *J. Exp. Med.*, 1925, xli, 535.
9. Neill, J. M., *J. Exp. Med.*, 1925, xli, 551.
10. Hoppe-Seyler, F., Handbuch der physiologisch- und patholigisch-chemischen Analyse, Berlin, 6th edition, 1893, 277, 280, 281.
11. Dittrich, P., *Arch. exp. Path. u. Pharmakol.*, 1891–92, xxix, 247, 251.
12. Conant, J. B., *J. Biol. Chem.*, 1923, lvii, 401.
13. Neill, J. M., and Avery, O. T., *J. Exp. Med.*, 1924, xxxix, 757.
14. Dogiel, J., *Centr. med. Wissensch.*, 1875, xiii, 499.

BIOLOGY OF BACTERIUM LEPISEPTICUM.

I. Effects of Oxygen Tension and the Presence of Rabbit Blood on Growth, Dissociation, and Virulence.

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During the course of our studies of the rabbit respiratory infection, snuffles—pneumonia—septicemia,[1] we have isolated many strains of the microbic incitant, *Bacterium lepisepticum*. The cultures have been obtained from the nasal passages of normal and infected rabbits, from the lungs and heart's blood of animals dying of pneumonia, and from subcutaneous abscesses and middle ear infections. They differ one from another in minor respects but all possess the main characteristics of the Pasteurella, rabbit septicemia group.

A common type of the organism, described by Theobald Smith in 1887,[2] has been studied more recently by De Kruif.[3] His cultures, obtained from rabbits dying of spontaneous pneumonia, were designated as Microbe D and were characterized as follows: "Microbe D grows diffusely in serum and plain broth, forms rather opaque, fluorescing colonies on serum agar, and is highly virulent for rabbits."[3] Its acid agglutination optimum lies between pH 3.0 and 3.5.

We are familiar with De Kruif's cultures of Microbe D and have found the type to be widespread in the nasal passages of normal and infected rabbits in different localities and in the pleural fluid, lungs, and heart's blood of rabbits dying of spontaneous pneumonia.

A dissociation of this microbe with the appearance of another form, Type G, has been described by De Kruif (Table I). The

[1] Webster, L. T., *J. Exp. Med.*, 1924, xxxix, 837; xl, 109.

[2] Smith, T., *J. Comp. Med. and Surg.*, 1887, viii, 24.

[3] De Kruif, P. H., *J. Exp. Med.*, 1921, xxxiii, 773.

phenomenon was stated to occur in extract broth cultures, to be favored by high concentrations of peptone, and to be inhibited by beef infusion or undiluted serum. The variant, Microbe G, showed a granular growth in fluid media, formed a translucent, bluish non-fluorescent colony, was of low virulence, and floculated best in buffer solutions of pH 4.1 to 4.7.[4] De Kruif noted the occurrence of both Microbes D and G in the nasal passages of normal rabbits.[5]

This D to G transformation has occurred from time to time in many of our stock cultures and we have recovered Microbe G from the nasal passages of normal and infected rabbits. Besides confirming the work of De Kruif, the studies described in this paper permit a more complete understanding of this dissociation phenomenon and sudden drop in virulence.[6]

TABLE I.

Distinguishing Characteristics of Bacterium lepisepticum, Types D and G.

Type.	Virulence.	Colony.	Broth.	Acid agglutination.
				pH
D	+++	Fluorescent.	Diffuse.	3.0–3.5
G	±	Non-fluorescent.	Granular.	3.5–4.7

Growth of Microbe D in Meat Extract Broth, pH 7.4.

Experiment 1.—A culture of *Bacterium lepisepticum*, Type D, was inoculated into meat extract broth, pH 7.4, the medium used throughout these experiments, unless otherwise designated. After 17 hours incubation, broth dilutions, 10^{-1} to 10^{-9}, were made and plated on blood agar to determine the number of organisms per cc. in the original culture. ½ cc. from each dilution was then transferred to a test-tube containing 4½ cc. of broth. The tubes were incubated and examined for growth after 24, 48, and 72 hours.

In Table II the results of this and several succeeding experiments are shown. And it can be seen that under the conditions of Experiment 1, growth occurred only in tubes which had received an inocula-

[4] De Kruif, P. H., *J. Exp. Med.*, 1922, xxxv, 561.

[5] De Kruif, P. H., *J. Exp. Med.*, 1922, xxxvi, 309.

[6] A preliminary report of this work has recently appeared (Webster, L. T., *Proc. Soc. Exp. Biol. and Med.*, 1924–25, xxii, 139).

tion of 100,000 or more bacteria. The character of this growth was then studied more carefully by a serial counting method described in Experiment 2.

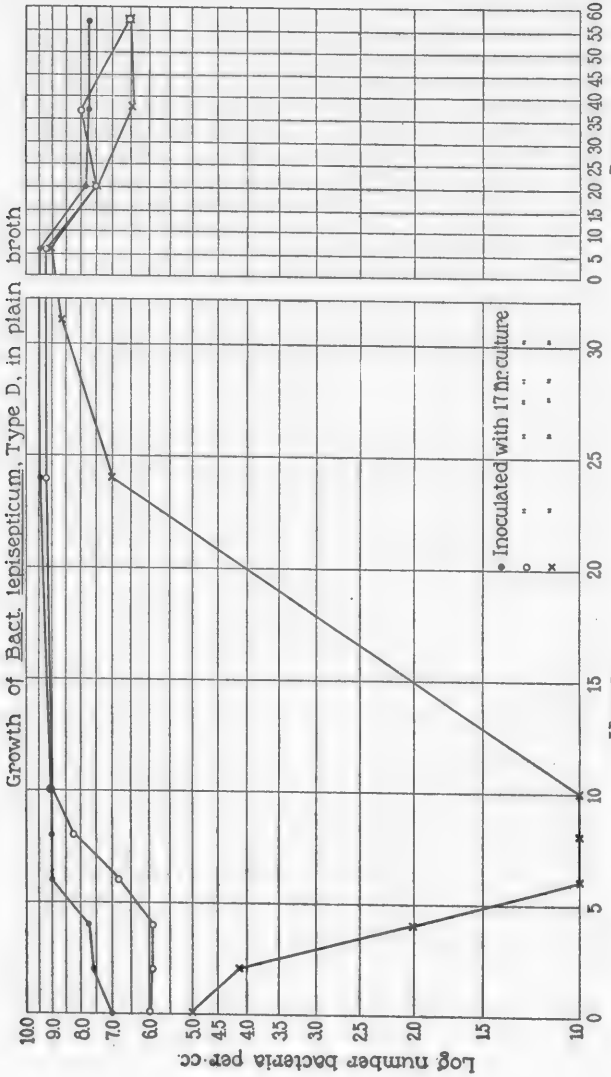
Experiment 2.—Three Erlenmeyer flasks of broth were inoculated with different quantities of a 17 hour broth culture of Microbe D and brought to a volume of 150 cc. Flask 1 received 10,000,000 per cc., Flask 2, 1,000,000, and Flask 3, 100,000. Immediately after inoculation and thereafter at hourly intervals, 1 cc. was removed from each flask and plated by the dilution method to estimate the number of living bacteria present.

TABLE II.

Growth Characteristics of Bacterium lepisepticum, Types D and G.

Experiment No.	Type.	Media.	No. of bacteria inoculated per tube.										D to G transformations.
			1,000,000,000	100,000,000	10,000,000	1,000,000	100,000	10,000	1,000	100	10	1	
1	D	Plain broth.	++	++	++	++	++	0	0	0	0	0	+++
3	G	" "	++	++	++	++	++	++	++	++	++	++	
5	D	" " + 0.6 per cent blood.	++	++	++	++	++	++	++	++	++	++	±
7	"	Plain broth + vaseline seal.	++	++	++	++	++	++	++	++	++	++	±
8	"	Plain broth + 0.00003 cc. blood.	++	++	++	++	++	++	++	++	++	++	±
9	"	Plain broth + 0.00003 cc. autoclaved blood.	++	++	++	++	++	++	++	++	++	++	±

The results of these counts are plotted in Text-fig. 1. The bacteria in Flask 1 increased in numbers immediately but slowly and reached a maximum of 1,000,000,000 per cc. in 6 hours. The number of organisms in Flask 2 remained stationary for 4 hours before a rise in the count was noted. And in Flask 3, the number per cc. fell rapidly to less than 10 and remained so for several hours, after which multiplication occurred and in 24 hours the maximum count of 1,000,000,000 was obtained. In other experiments still smaller inoculations resulted in a rapid drop to zero in the count with no subsequent rise.



TEXT-FIG. 1. Growth of *Bacterium lepisepticum*, Type D, in flasks of extract broth, pH 7.4.

When flasks were seeded with cultures in lag, the stationary phase continued for some hours but when sufficient numbers of bacteria in the period of logarithmic growth were inoculated, multiplication continued at the same rate until the maximum count of 1,000,000,000 per cc. was reached.

These observations are quite similar to those of Chesney on the growth curve of pneumococcus.[7]

Growth of Microbe G in Meat Extract Broth, pH 7.4.

The behavior in broth of Microbe G, the so called type variant, was compared with that of the original strain, Type D, by tests similar to those described above; and it was found that a 16 hour culture seeded quantitatively into a series of nine broth tubes grew well in each instance. Inoculations of 1 or 2 organisms to 1,000,000,000 multiplied freely (Experiment 3, Table II). Furthermore, when frequent counts were made, it was found that a very few organisms, 18, 6, and $1\frac{1}{2}$ hours old, when transferred to fresh media, began immediately to multiply without lag and continued to increase logarithmically until a maximum number of 1 billion per cc. was reached (Text-fig. 2).

Evidently then, meat extract broth under aerobic conditions is adequate for the optimum growth of Microbe G, the form of low virulence, but not for Type D, the highly virulent strain.

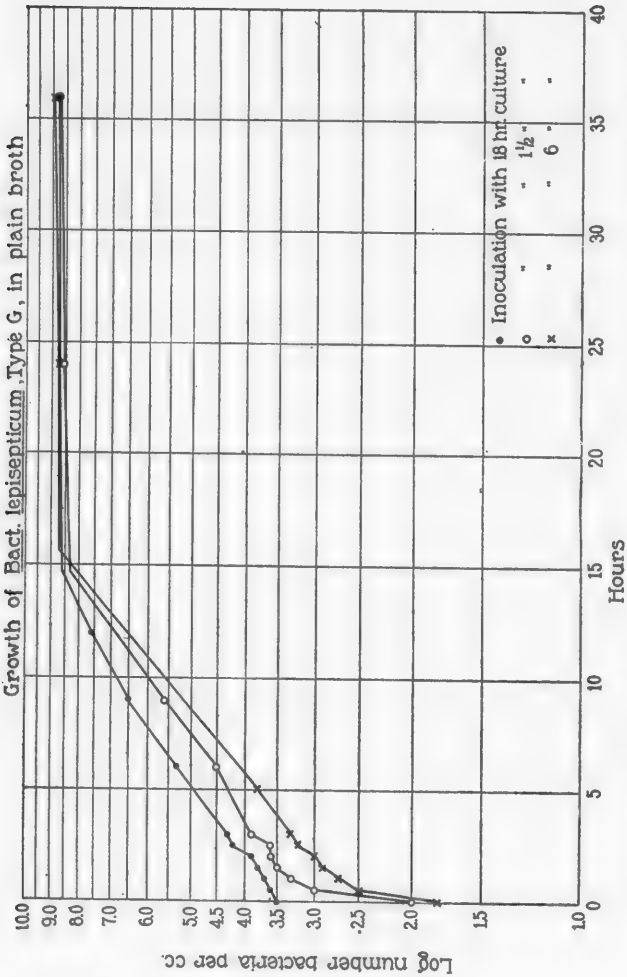
Growth of Microbes D and G in Extract Broth Plus Rabbit Blood.

Rabbit blood added to extract broth modified the growth of Microbe D in a striking manner.

Experiment 5.—Nine tubes containing 0.6 per cent citrated blood in 5 cc. of broth were inoculated with nine dilutions from 1:1 to 1:1,000,000,000, respectively, of a 16 hour bouillon culture of Microbe D. Certain dilutions were plated to determine the actual numbers of organisms present; and after 24 hours incubation, the number of organisms on the plates was counted and the tubes inspected.

A heavy growth occurred invariably in each tube (Table II, Experiment 5). The smallest number of organisms employed multiplied freely in this medium.

[7] Chesney, A. M., *J. Exp. Med.*, 1916, xxiv, 387.



TEXT-FIG. 2. Growth of *Bacterium lepisepticum*, Type G, in flasks of extract broth, pH 7.4.

A further analysis of this growth was carried out by means of serial counts as in Experiment 2.

Experiment 6.—An Erlenmeyer flask containing 150 cc. of broth plus 0.6 per cent rabbit blood was inoculated with 1 cc. of a 1:100,000 dilution of a 17 hour broth culture of Microbe D and incubated at 37°. At ½ or 1 hour intervals thereafter, 1 cc. was removed and counted by the dilution and plating method. After 1½ hours incubation, 20 cc. of culture was removed from Flask 1 and put into a second flask of similar blood broth. This new culture was then incubated and submitted to frequent counts. 4½ hours later, or 6 hours after inoculation, 1 cc. of a 1:100 dilution was again taken from Flask 1 and placed into a third flask of the same medium. Counts were begun on this fresh culture and were continued throughout the day on all three flasks.

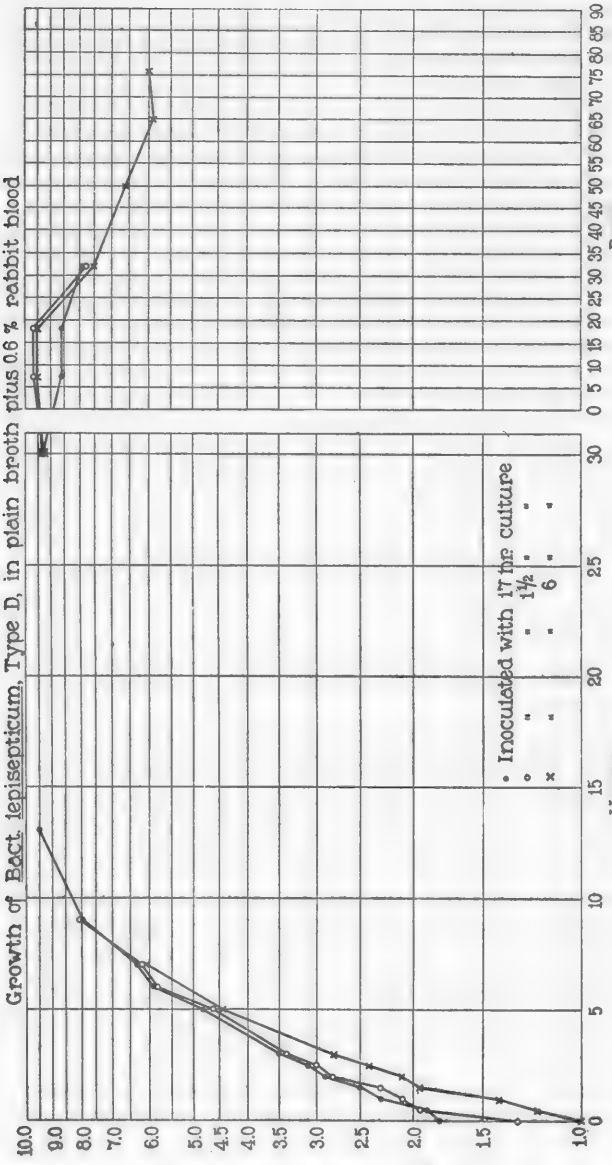
The growth of the bacteria in Flask 1 represents, therefore, the activity of a 17 hour culture of Microbe D in fresh blood broth media, that of Flask 2, the activity of a 1½ hour culture, and of Flask 3, a 6 hour culture under similar conditions. From Text-fig. 3, in which the counts are plotted, it may be seen that Flask 1 received an inoculation of 65 organisms, Flask 2, 21, and Flask 3, 6. In each instance a logarithmic increase in the number per cc. began immediately and continued for about 10 hours. When the count of 1,000,000,000 per cc. had been reached, no further increase was noted. For several weeks the counts remained about the same; then a slow and progressive drop occurred.

In brief, then, the addition of rabbit blood to extract broth furnishes optimum growth conditions for Type D by enabling the smallest number of organisms to multiply logarithmically without lag in a manner similar to the multiplication of Microbe G in plain broth.

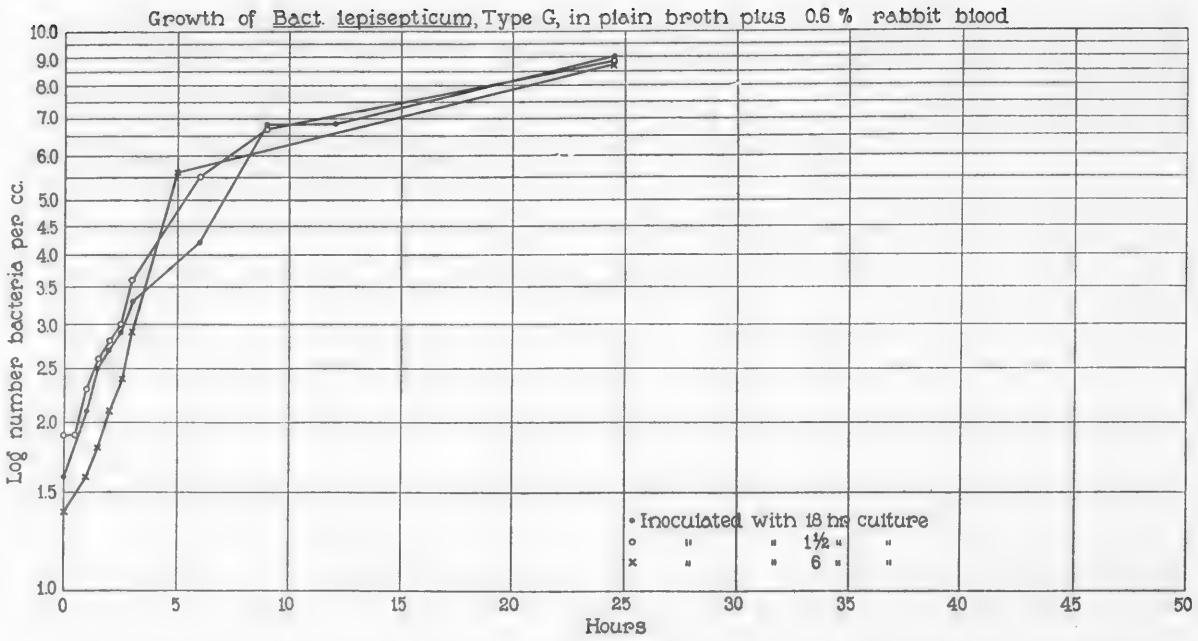
The behavior of Type G in blood broth did not differ essentially from that in plain broth (Text-fig. 4). Small inoculations were sufficient for growth, multiplication began immediately and continued logarithmically for 8 to 10 hours until a maximum number of about 500,000,000 was reached.

Dissociation of Bacterium D to Type G.

If broth tubes are inoculated with Type D and incubated for 48 hours or more as in Experiment 1, a scum or granular film appears at the surface of the liquid. When the tube is shaken, this material



TEXT-FIG. 3. Growth of *Bacterium lepisepticum*, Type D, in flasks of extract broth plus 0.6 per cent rabbit blood.



TEXT-FIG. 4. Growth of *Bacterium lepisepticum*, Type G, in flasks of extract broth plus 0.6 per cent rabbit blood.

settles and a new scum forms in a few hours. This scum proved to be made up of aggregations of Type G bacteria.

Apparently this change from Microbe D to G was occurring at the surface of the fluid where the oxygen tension was at atmospheric pressure and not in the depths of the media where methylene blue was rapidly decolorized. The following experiment was planned to test the influence of media at low oxygen tension on this dissociation phenomenon.

Experiment 7.—Tubes containing 5 cc. of meat extract broth were placed in boiling water for ½ hour, layered with vaseline at the surface of the fluid, and allowed to cool. Each tube was then inoculated with ½ cc. of a graded dilution from 1:1 to 1:1,000,000,000 of a 17 hour culture of Microbe D and incubated at 37°.

An abundant growth was present in all tubes 24 hours later (Table II, Experiment 7). And subsequently, for a 3 week period of observation, no D to G transformation occurred.

Later it was noticed that the D to G dissociation in broth tubes containing blood was strongly inhibited. So a conclusion was reached that a reduction in oxygen tension of extract broth or the addition of blood to this medium furnished conditions for the optimum growth of the virulent Microbe D and retarded the appearance of the variant G forms of low virulence.

Growth of Microbe D in Broth Plus Minute Amounts of Rabbit Blood.

We were impressed with the similarity of these results to those in certain experiments with pneumococcus published by Avery and his associates.[8] They believe that two substances are necessary for the optimum growth of pneumococci, a heat-labile vitamine-like factor, and a heat-stabile catalyst, probably of peroxidase nature. Both of these factors are present in blood and in fresh potato and when added to extract broth enable small numbers of bacteria to multiply freely without lag. They prepared an "artificial" peroxidase with gum arabic and ferrous sulfate which when combined with

[8] Avery, O. T., *et al.*, *J. Exp. Med.*, 1921–24, xxxiv–xl; *Proc. Soc. Exp. Biol. and Med.*, 1921, xviii–xix.

yeast extract and added to broth induced good growth of pneumococci. These experiments together with the researches of McLeod and Gordon[9] have given rise to the theory that certain bacteria which evolve peroxides require for active growth in aerobic media the presence of a peroxidase to split up these toxic substances.

It seemed to us quite possible that in our experiments blood was functioning in a similar manner. So tests were applied to determine the reactivity of the blood in high dilutions before and after autoclaving.

Experiment 8.—Tubes of broth plus 0.00003 cc. of rabbit blood were inoculated with a broth culture of Microbe D in dilutions increasing exponentially from 1 to 9.

Growth occurred promptly in every tube (Table II, Experiment 8), and for some weeks little or no D to G transformation occurred. The benzidine reaction in this medium was positive and further experiments showed that optimum growth without any dissociation was always obtained in broth to which sufficient blood had been added to give a positive benzidine test.

When flasks containing 150 cc. of broth plus 0.0003 per cent blood were inoculated with small numbers of 17, 6, and $1\frac{1}{2}$ hour cultures of Microbe D, precisely as in Experiment 6, and counted at short intervals thereafter, figures were obtained which showed in each case immediate logarithmic multiplication with a maximum number of 1,000,000,000 per cc. in 9 to 10 hours.

These results, plotted in Text-fig. 5, are very similar to those in Text-fig. 3 and show that very small amounts of blood, to the limit of the benzidine reaction, function just as well as large quantities in inducing optimum growth and permanence of type characteristics.

Experiment 9.—Autoclaved blood in similar small quantities, 0.0003 per cent, just sufficient to give a positive benzidine test, was added to test-tubes and to Erlenmeyer flasks. Microbe D was added to the tubes in various dilutions as in Experiment 8, and to flasks as in Experiment 6. Counts were made and the flasks and tubes were inspected after 1, 2, and 3 days and after 1, 2, and 3 weeks incubation.

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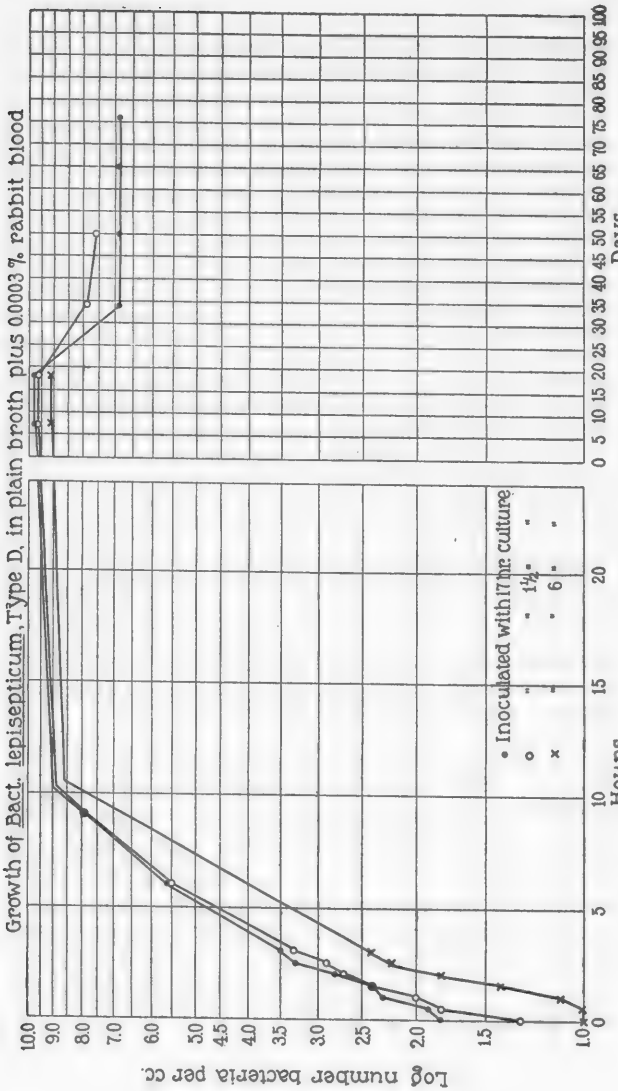
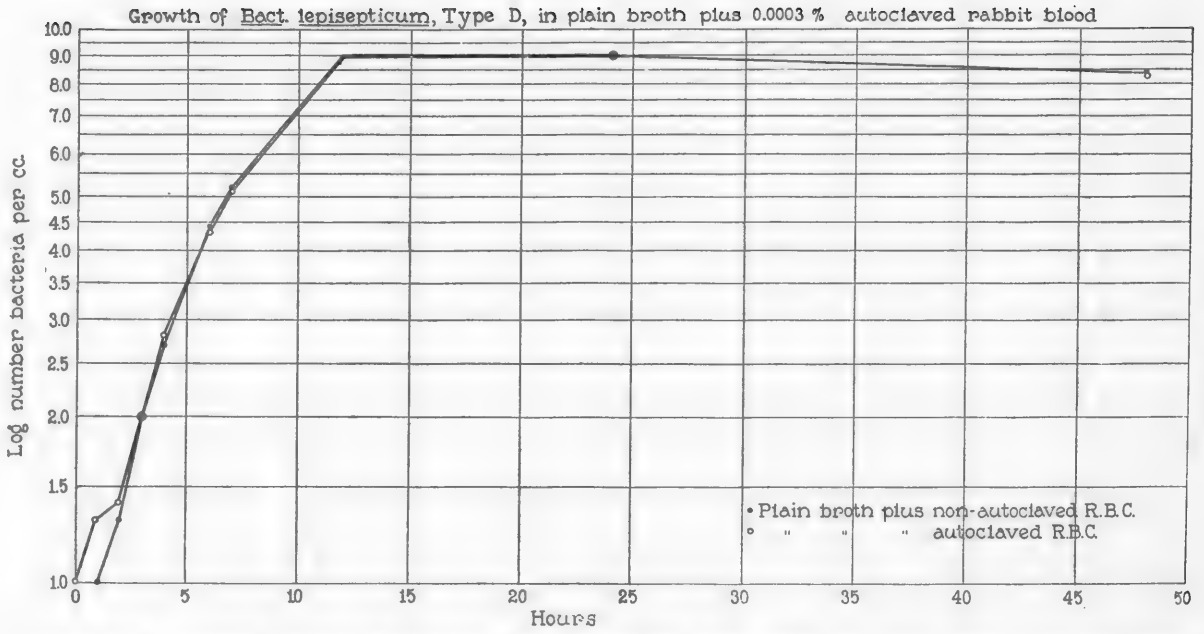
[9] McLeod, J. W., and Gordon, J., *J. Path. and Bact.*, 1922–23, xxv–xxvi.



TEXT-FIG. 5. Growth of *Bacterium lepisepticum*, Type D, in flasks of extract broth plus 0.0003 per cent rabbit blood.



TEXT-FIG. 6. Growth of *Bacterium lepisepticum*, Type D, in flasks of extract broth plus 0.0003 per cent autoclaved blood.

Good growth occurred in all tubes (Table II, Experiment 9) and the 17, 6, and 1½ hour cultures in the flasks although inoculated in very small number, multiplied logarithmically (Text-fig. 6) and behaved similarly to the cultures in Experiment 6 (Text-fig. 3).

DISCUSSION.

These experiments show that virulent types of *Bacterium lepisepticum* require for optimum growth and maintenance of type purity and pathogenicity either a lowered oxygen tension in a general nutrient medium or, under atmospheric conditions, the presence of an accessory substance found in blood. This substance is active in high dilutions to the limit of the benzidine reaction, and is heat-stabile. Probably, therefore, it is not an ordinary food or of vitamine nature. Further experiments on this phase of the problem will be published shortly.

The apparent disagreement of De Kruif's experiments which showed that meat infusion and serum inhibited the D to G dissociation and that high concentrations of peptone with decreasing amounts of meat infusion favored the process is explainable by the presence of red cells or derivatives therefrom in the serum and meat infusion used in those experiments.

CONCLUSIONS.

1. *Bacterium lepisepticum*, Type D, inoculated in small amounts into meat extract broth, pH 7.4, under aerobic conditions failed to grow. Larger inoculations underwent a period of lag before the logarithmic growth phase began.

2. In this medium, dissociation of Microbe D to the variant G occurred readily.

3. A lowered oxygen tension in this broth resulted in growth of small numbers of Type D with no lag and great inhibition of the type dissociation process.

4. Similar optimum growth conditions were obtained by adding to extract broth, under aerobic conditions, autoclaved or unautoclaved rabbit blood in very small amounts, 0.0003 per cent, just sufficient to give a positive benzidine test.

5. The nature of this accessory substance is discussed.
6. The variant Type G grows equally well in plain or blood broth.

I wish to thank Mr. C. G. Burn for his assistance with the technical part of this work.